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ANÁLISE FOSFOPROTEÔMICA E GENÔMICA FUNCIONAL DE LINHAGENS DE *Leishmania* spp. SENSÍVEIS E RESISTENTES AO ANTIMÔNIO TRIVALENTE

por

Douglas de Souza Moreira

Belo Horizonte
2017

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Banca Examinadora:

Prof. Dra. Silvane Maria Fonseca Murta (CPqRR/FIOCRUZ) Presidente
Prof. Dr. Edward José de Oliveira (CPqRR/FIOCRUZ) Titular
Prof. Dra. Rosiane Aparecida da Silva Pereira (CPqRR/FIOCRUZ) Titular
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Prof. Dra. Patrícia Cuervo Escobar (IOC/FIOCRUZ) Titular
Prof. Dr. Elio Hideo Babá (CPqRR/FIOCRUZ) Suplente

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.”

José de Alencar

Dedico esta tese aos meus pais, que sempre foram o meu alicerce.

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RESUMO

A leishmaniose é um complexo de doenças com ampla diversidade epidemiológica e clínica causada por protozoários parasitas pertencentes ao gênero *Leishmania*. A fosforilação de proteínas é uma das modificações pós-traducionais mais estudadas, que está envolvida em diferentes eventos celulares em *Leishmania*. Na primeira parte desse estudo, nós realizamos uma análise fosfoproteômica comparativa de linhagens de *L. braziliensis* sensível e resistente ao antimônio trivalente (Sb^{III}), utilizando eletroforese em gel diferencial bidimensional (2D-DIGE) seguida por espectrometria de massas. Para investigar a abundância diferencial de fosfoproteínas associada com resposta ao estresse induzido à droga e mecanismos de resistência ao Sb^{III}, nós comparamos amostras não tratadas e tratadas com Sb^{III} de cada linhagem. Análises comparativas revelaram um total de 116 *spots* que apresentaram diferença estatisticamente significativa na abundância de fosfoproteínas, incluindo 11 e 34 *spots* especificamente correlacionados com estresse devido ao tratamento com a droga e resistência ao Sb^{III}, respectivamente. Foram identificadas 48 proteínas diferentes distribuídas em sete categorias de processos biológicos. A categoria "enovelamento de proteínas/chaperonas e resposta ao estresse" está envolvida principalmente em resposta ao estresse com Sb^{III}, enquanto que as categorias "antioxidante/detoxificação", "processos metabólicos", "processamento de RNA/DNA" e "biossíntese de proteínas" estão moduladas no caso de resistência à droga. Alinhamentos de sequências múltiplas foram realizados para validar a conservação de resíduos fosforilados em nove proteínas identificadas nesse estudo. Ensaios de Western blot foram conduzidos para validar a análise quantitativa do fosfoproteoma. Os resultados mostraram níveis de expressão diferencial de três fosfoproteínas nas linhagens analisadas. Na segunda parte desse estudo, análises de Western blot demonstraram que as proteínas nucleosídeo difosfato quinase b (NDKb) e fator de elongação 2 (EF2) estão mais e menos expressas, respectivamente, na linhagem de *L. braziliensis* resistente ao Sb^{III}, corroborando nossos dados anteriores do fosfoproteoma. NDKb é responsável pela síntese de nucleosídeos trifosfatos e tem papel chave no metabolismo de purina em protozoários tripanossomatídeos. EF2 é um importante fator para síntese de proteínas. A superexpressão dos genes *NDKb* e *EF2* nas espécies *L. braziliensis* e *L. infantum* foi realizada para investigar a contribuição destas proteínas no fenótipo de resistência ao Sb^{III}. As linhagens de *L. braziliensis* superexpressoras de NDKb ou EF2 foram 1,6 a 2,1 vezes mais resistentes ao Sb^{III} do que a linhagem sensível não transfectada. Em contraste, nenhuma diferença na susceptibilidade ao Sb^{III} foi observada em *L. infantum* superexpressora de NDKb ou EF2. Ensaios de susceptibilidade mostraram que as linhagens de *L. braziliensis* superexpressoras de NDKb apresentaram elevada resistência à lamivudina, um agente antiviral, mas esta droga não alterou a atividade leishmanicida em associação com Sb^{III}. O clone de *L. braziliensis* superexpressor de EF2 foi 1,2 vezes mais resistente ao inibidor da quinase de EF2 do que a linhagem sensível. Surpreendentemente, este inibidor aumentou o efeito leishmanicida do Sb^{III}, sugerindo que esta associação pode ser uma estratégia valiosa para a quimioterapia das leishmanioses. Portanto, esse novo estudo nos permitiu determinar o perfil do fosfoproteoma de *L. braziliensis*, identificando alguns candidatos potenciais para redes bioquímicas ou de sinalização associadas com o fenótipo de resistência ao Sb^{III} neste parasito. Além disso, nossos resultados representam o primeiro estudo de superexpressão dos genes *NDKb* e *EF2* que demonstra um aumento de resistência ao Sb^{III} em *L. braziliensis*, o que pode contribuir para o desenvolvimento de novas estratégias para o tratamento das leishmanioses.

Palavras-chave: *Leishmania* spp., resistência ao antimônio, análise fosfoproteômica, nucleosídeo difosfato quinase b, fator de elongação 2.

ABSTRACT

Leishmaniasis is a disease complex with wide epidemiological and clinical diversity caused by protozoan parasites belonging to the genus *Leishmania*. Protein phosphorylation is one of the most studied post-translational modifications that is involved in different cellular events in *Leishmania*. In the first part of this study, we performed a comparative phosphoproteomics analysis of antimony (Sb^{III})-resistant and -susceptible lines of *L. braziliensis* using a 2D-DIGE (two dimensional differential gel electrophoresis) approach followed by mass spectrometry. In order to investigate the differential phosphoprotein abundance associated with the drug-induced stress response and Sb^{III}-resistance mechanisms, we compared non-treated and Sb^{III}-treated samples of each line. Pair wise comparisons revealed a total of 116 spots that showed a statistically significant difference in phosphoprotein abundance, including 11 and 34 spots specifically correlated with drug treatment and resistance, respectively. We identified 48 different proteins distributed into seven biological process categories. The category “protein folding/chaperones and stress response” is mainly implicated in response to Sb^{III} treatment, while the categories “antioxidant/detoxification”, “metabolic process”, “RNA/DNA processing” and “protein biosynthesis” are modulated in the case of antimony resistance. Multiple sequence alignments were performed to validate the conservation of phosphorylated residues in nine proteins identified here. Western blot assays were carried out to validate the quantitative phosphoproteome analysis. The results revealed differential expression level of three phosphoproteins in the lines analyzed. In the second part of this study, Western blot analysis demonstrated that nucleoside diphosphate kinase b (NDKb) and elongation factor 2 (EF2) proteins are more and less expressed, respectively, in Sb^{III}-resistant line of *L. braziliensis*, corroborating our previous phosphoproteomic data. NDKb is responsible for nucleoside triphosphates synthesis and it has key role in the purine metabolism in trypanosomatid protozoa. EF2 is an important factor for protein synthesis. Overexpression of *NDKb* and *EF2* genes in *L. braziliensis* and *L. infantum* species was performed to investigate the contribution of these proteins in Sb^{III}-resistance phenotype. NDKb or EF2-overexpressing *L. braziliensis* lines were 1.6 to 2.1-fold more resistant to Sb^{III} than the untransfected wild-type line (WTS). In contrast, no difference in Sb^{III} susceptibility was observed in *L. infantum* parasites overexpressing NDKb or EF2. Susceptibility assays showed that NDKb-overexpressing *L. braziliensis* lines presented elevated resistance to lamivudine, an antiviral agent, but it did not alter the leishmanicidal activity in association with Sb^{III}. EF2-overexpressing *L. braziliensis* clone was 1.2-fold more resistant to EF2 kinase inhibitor than the WTS line. Surprisingly, this inhibitor increased the antileishmanial effect of Sb^{III}, suggesting that this association might be a valuable strategy for leishmaniasis chemotherapy. Therefore, this novel study allowed us to profile the *L. braziliensis* phosphoproteome, identifying several potential candidates for biochemical or signaling networks associated with antimony resistance phenotype in this parasite. Furthermore, our findings represent the first study of *NDKb* and *EF2* genes overexpression that demonstrates an increase of Sb^{III} resistance in *L. braziliensis* which can contribute to develop new strategies for leishmaniasis treatment.

Keywords: *Leishmania* spp., antimony resistance, phosphoproteomic analysis, nucleoside diphosphate kinase b, elongation factor 2.

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LISTA DE QUADROS

LISTA DE SIGLAS E ABREVIATURAS

2D-DIGE – eletroforese em gel diferencial bidimensional

2DE – eletroforese bidimensional

ABC – *ATP-binding cassette*

ACR2 – antimoniato redutase 2

AG – adenina-guanina

AQP1 – aquagliceroporina 1

ARGG/ASS – argininosuccinato sintetase

CTP – citidina trifosfato

Cy – cianina

Cys – cisteína

EIF-1A – fator de iniciação da tradução 1A

ERNs – espécies reativas de nitrogênio

EROs – espécies reativas de oxigênio

FACS – *fluorescence-activated cell sorting*

FeSOD – ferro superóxido dismutase

GSH – glutationa

GTP – guanosina-5'-trifosfato

H_2O_2 – peróxido de hidrogênio

HSP – proteína de choque térmico

kDNA – DNA do cinetoplasto

KMP-11 – proteína de membrana dos kinetoplastídeos-11

LbSbR – linhagem de *Leishmania (Viannia) braziliensis* resistente ao Sb^{III}

LbSbR 0.025 – amostra resistente tratada com 0,025 mg/mL de Sb^{III}

LbSbR 2 – amostra resistente tratada com 2 mg/mL de Sb^{III}

LbWTS – linhagem de *Leishmania (Viannia) braziliensis* sensível ao Sb^{III}

LbWTS 0 – amostra sensível sem tratamento com Sb^{III}

LbWTS 0.025 – amostra sensível tratada com 0,025 mg/mL de Sb^{III}

LC – leishmaniose cutânea

LMC – leishmaniose mucocutânea

LV – leishmaniose visceral

MAPK – *mitogen-activated protein kinase*

MPTs – modificações pós-traducionais

mRNA – RNA mensageiro

MRPA – proteína de resistência a múltiplas drogas A

MS – Ministério da Saúde

NDK – nucleosídeo difosfato quinase

NDKb – nucleosídeo difosfato quinase b

NDP – nucleosídeo difosfato

NTP – nucleosídeo trifosfato

O_2^- – ânion superóxido

ODC – ornitina decarboxilase

OH^- – radical hidroxila

OPAS/OMS – Organização Panamericana da Saúde/Organização Mundial da Saúde

PCNA – antígeno nuclear de proliferação celular

PGP – fosfoglicoproteína

PGPA – fosfoglicoproteína A

PTR1 – pteridina redutase 1

Sb^{III} – antimônio trivalente

Sb-TS – complexo antimônio-tiol

Sb^{V} – antimônio pentavalente

SF – motivo serina-fenilalanina

SL – sequência líder ou miniexon

SOD – superóxido dismutase

STI1 – proteína induzida por estresse

TDR1 – redutase dependente de tiol 1

TiO_2 – dióxido de titânio

$T(SH)_2$ – tripanotiona

TXNPx – triparedoxina peroxidase

UTP – uridina-5’-trifosfato

WHO – Organização Mundial da Saúde

γ-GCS – γ-glutamilcisteína sintetase

SUMÁRIO

1 INTRODUÇÃO

1.1 Epidemiologia e formas clínicas das leishmanioses

As leishmanioses representam um grupo de doenças causadas por diferentes espécies de protozoários parasitas do gênero *Leishmania* (Ordem Kinetoplastida; Família Trypanosomatidae). Os protozoários dessa família são organismos unicelulares flagelados que apresentam o cinetoplasto, que consiste no DNA mitocondrial ou DNA do cinetoplasto (kDNA), presente na mitocôndria única desses parasitos. O cinetoplasto está situado próximo à base do flagelo e ele é composto por maxicírculos e minicírculos de DNA concatenados e, assim como mitocôndrias de outros eucariotos, o kDNA contém sequências de RNA ribossomal e de componentes de complexos respiratórios (Balaña-Fouce et al., 1998).

As leishmanioses têm sido consideradas como uma das doenças mais negligenciadas, ocupando o segundo lugar em mortalidade e o quarto lugar em morbidade entre as infecções tropicais (Bern et al., 2008). Elas são um problema de saúde pública em muitos países em desenvolvimento, principalmente na África, Ásia, Mediterrâneo, América Latina e Oriente Médio (WHO, 2016). Atualmente, estima-se que 12 milhões de indivíduos estejam infectados com *Leishmania*, e que mais de 350 milhões de pessoas vivam em áreas de risco de infecção da doença (Alvar et al., 2012). As leishmanioses são endêmicas em 98 países, com cerca de 700.000 a 1 milhão de novos casos notificados a cada ano, e uma estimativa de 20.000 a 30.000 mortes anuais (WHO, 2017).

No Brasil, o Ministério da Saúde (MS) estima que, anualmente, cerca de três mil indivíduos sejam infectados pela doença. Entre 1992 e 2011, o país respondeu por 90% das 600 mil ocorrências registradas em toda a América Latina. Entre 2000 e 2011, foram registradas mais de 2,7 mil mortes por leishmanioses no Brasil. Os maiores índices de mortalidade foram registrados nos estados do Pará, Tocantins, Maranhão, Piauí, Ceará, São Paulo, Bahia e em Minas Gerais. Atualmente, há casos da doença nas 27 Unidades da Federação (Agência Brasil, 2013).

O gênero *Leishmania* compreende cerca de 30 espécies de protozoários morfologicamente semelhantes, entre as quais 21 são capazes de infectar humanos. As três formas principais da doença são: cutânea, mucocutânea e visceral (Fig. 1) (WHO, 2017). Essas manifestações clínicas abrangem uma diversidade de sinais,

sintomas, dependem de fatores genéticos e da resposta imune do hospedeiro e estão relacionadas à espécie do parasito, diferindo em distribuição geográfica, hospedeiros e vetores envolvidos, taxas de incidência e de mortalidade (Ashford et al., 1992; Herwaldt, 1999; Murray et al., 2005; Reithinger, 2007).

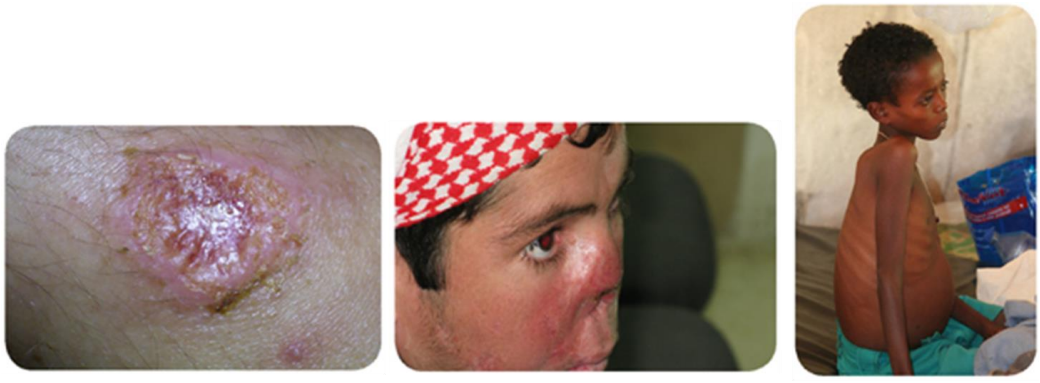

Figura 1 – Principais formas clínicas da doença: cutânea (A), mucocutânea (B) e visceral (C) (Google Imagens).

A leishmaniose cutânea (LC) é a forma mais comum e menos grave da doença. É caracterizada por lesões na pele das partes expostas do corpo, como face, braços e pernas, que se evoluem ao longo de semanas a meses, podendo deixar cicatrizes permanentes (Fig. 1A). LC pode ser causada por diversas espécies de *Leishmania*, como *L. major*, *L. tropica* e *L. aethiopica* no Velho Mundo, e *L. mexicana*, *L. amazonensis*, *L. guyanensis*, *L. panamensis*, *L. braziliensis* e *L. peruviana* no Novo Mundo (Reithinger, 2007; Goto & Lauletta Lindoso, 2012).

A LC é mais amplamente distribuída, sendo que 95% dos casos ocorrem nas Américas, na bacia do Mediterrâneo, no Oriente Médio e na Ásia Central (WHO, 2017). Afeganistão, Argélia, Colômbia, Brasil, Irã e Síria são os países com maior número de casos da doença, respondendo por cerca de 70 a 75% da incidência global estimada de LC (Fig. 2A). Estima-se que 0,6 a 1 milhão de novos casos de LC ocorram anualmente em todo o mundo (WHO, 2017).

A leishmaniose mucocutânea (LMC) resulta na destruição parcial ou total das membranas mucosas do nariz, boca e garganta (Fig. 1B). É causada por *L. braziliensis* e *L. panamensis*, com cerca de 90% dos casos relatados no Brasil, Peru, Etiópia e Bolívia (WHO, 2017).

A leishmaniose visceral (LV), também conhecida como calazar, é a forma mais grave da doença, que pode ser letal se não for tratada. Caracteriza-se por febre irregular, fraqueza, anorexia, substancial perda de peso, anemia e hepatoesplenomegalia (Fig. 1C). No Velho Mundo, é causada por *L. donovani* e *L. infantum* (conhecida como *L. chagasi* no Novo Mundo) (Salam et al., 2014).

A LV é altamente endêmica no subcontinente indiano e no leste da África. Estima-se que 50.000 a 90.000 novos casos da doença ocorram mundialmente a cada ano. Em 2015, mais de 90% dos novos casos relatados ocorreram na Índia, Somália, Sudão, Sul do Sudão, Etiópia, Brasil e Quênia (Fig. 2B) (WHO, 2017). No Brasil, a ocorrência de LV era inicialmente limitada a áreas rurais e pequenas localidades urbanas, todavia nas últimas décadas houve uma expansão para os grandes centros urbanos, tornando um grande problema de saúde pública em todo o país (WHO, 2010; Harhay et al., 2011).

Durante o período de 2001 a 2014 foi relatado um total de 797.849 casos novos de LC e LMC com média anual de 56.989 distribuídos em 17 dos 18 países endêmicos das Américas (OPAS/OMS, 2016). Nesse mesmo período, foram registrados 48.720 casos de LV com média anual de 3.480 casos, sendo a maioria deles (46.976) concentrados no Brasil. Em 2014, foram confirmados 19.402, 3.453 e 1.016 casos de LC, LV e LMC no Brasil, respectivamente (OPAS/OMS, 2016).

1.2 Morfologia e ciclo biológico de *Leishmania*

O parasito possui duas formas morfológicas e bioquimicamente distintas: amastigota e promastigota. As formas amastigotas (Fig. 3A) são arredondadas, sem flagelo aparente, que infectam e se multiplicam em células do sistema mononuclear fagocitário do hospedeiro vertebrado. As formas promastigotas (Fig. 3B) são alongadas, flageladas, móveis e vivem no lúmen do tubo digestivo do flebotomíneo (Balaña-Fouce et al., 1998).

Os parasitos do gênero *Leishmania* possuem um ciclo de vida digenético ou heteroxênico, cujas formas de desenvolvimento alternam-se entre hospedeiros invertebrados e mamíferos. Os hospedeiros invertebrados (insetos vetores) são fêmeas da Ordem Diptera, família Psychodidae, subfamília Phlebotominae, gênero *Lutzomyia* (Novo Mundo) e *Phlebotomus* (Velho Mundo) (Bates, 1994).

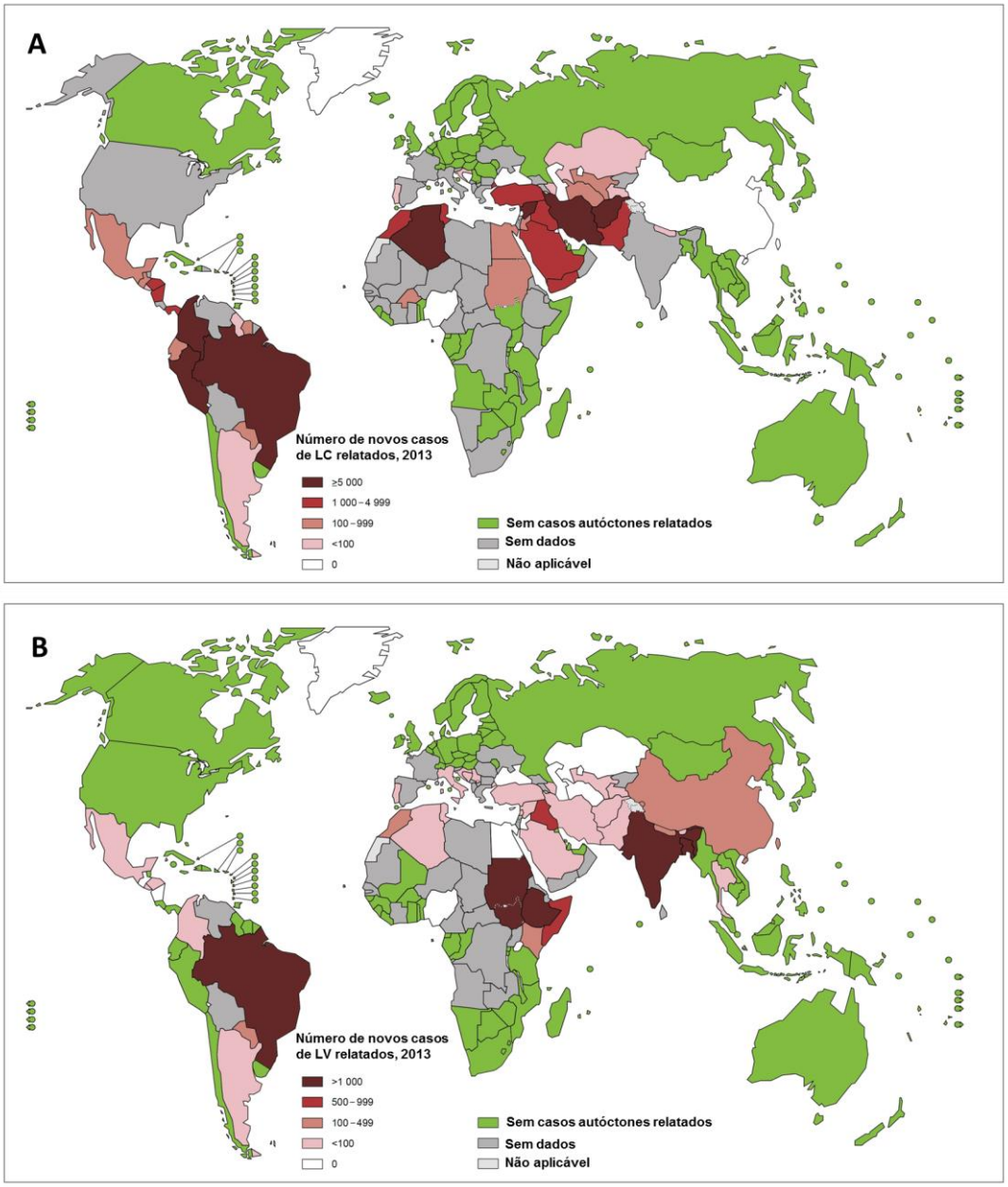

Figura 2 – Endemicidade mundial de leishmaniose cutânea (A) e de leishmaniose visceral (B) em 2013 (WHO, 2017).

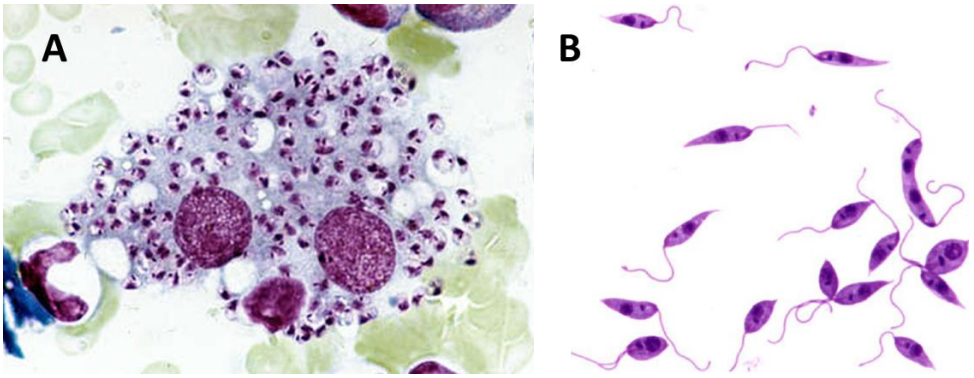

Figura 3 – Formas amastigotas (A) e promastigotas (B) de *Leishmania* spp. (Google Imagens).

O ciclo de vida do parasito inicia-se quando um flebotomíneo infectado com *Leishmania* spp. pica um humano ou outro hospedeiro mamífero para alimentar-se de sangue. Durante a picada, o inseto injeta a saliva que impede a coagulação sanguínea (Ribeiro et al., 1986). Após a ingestão de sangue, as formas promastigotas metacíclicas são liberadas e entram na pele do hospedeiro através de regurgitação (Turco & Descoteaux, 1992). A saliva do inseto contém fatores químicos que potencializam o poder infectivo do parasito e induzem uma rápida infiltração de neutrófilos e recrutamento substancial de macrófagos para o local da picada (Peters et al., 2008). Outros tipos celulares, como células de Langerhans e fibroblastos também podem ser infectados (Peters et al., 2008). A interação com as células do hospedeiro, principalmente neutrófilos e macrófagos, envolve reconhecimento e adesão, seguida por sinalização e invasão (Handman & Bullen, 2002). Os neutrófilos desempenham um papel importante, atuando como um "Cavalo de Troia" (Peters et al., 2008). Isso significa que, ao entrar em apoptose, os neutrófilos infectados induzem o recrutamento de monócitos, o que leva os neutrófilos apoptóticos a serem fagocitados e à entrada "silenciosa" dos parasitos nos macrófagos. Estes, por sua vez, são importantes para o estabelecimento final e amplificação da infecção. A adesão do parasito à superfície da célula hospedeira envolve o reconhecimento de moléculas expostas na superfície do parasito, como lipofosfoglicanos (Turco & Descoteaux, 1992) e a glicoproteína gp63 (Russell & Wilhelm, 1986). Estas moléculas ligam-se a diferentes receptores encontrados na superfície dos macrófagos, como receptores do complemento (CR1 e CR3), receptores de manose e receptores de fibronectina (Ueno & Wilson, 2012). Dessa maneira, o parasito é internalizado em um vacúolo parasitóforo (bolsa que abriga o parasito no interior de macrófagos), que a seguir funde-se com lisossomo, formando o fagolisossomo. Entretanto, as formas promastigotas metacíclicas podem retardar a formação do fagolisossomo, e a protease gp63 pode atuar degradando as enzimas lisossomais. Assim, as formas promastigotas metacíclicas transformam-se em amastigotas, que se multiplicam intensamente (Stuart et al., 2008). Posteriormente, ocorre o rompimento da membrana da célula e a liberação das formas amastigotas, as quais podem invadir novas células ou serem ingeridas por uma nova fêmea de flebotomíneo durante o seu repasto sanguíneo. É importante ressaltar que para espécies dermotrópicas de *Leishmania*, a lesão permanece na pele, mas para

Leishmania viscerotrópica, o parasito dissemina-se a partir do sítio da infecção inicial da pele para o fígado, baço e medula óssea (Ponte-Sucre, 2003).

Quando um novo flebotomíneo pica um hospedeiro vertebrado infectado, ele ingere sangue contendo macrófagos parasitados por amastigotas de *Leishmania*. A refeição sanguínea é digerida no intestino médio do inseto. Neste novo ambiente, as formas amastigotas são agrupadas e envolvidas por uma estrutura chamada de matriz peritrófica, que protege contra a ação de enzimas digestivas (Pimenta et al., 1997; Secundino et al., 2005). Posteriormente, ocorre o rompimento dos macrófagos e a liberação dos parasitos, que se diferenciam em promastigotas procíclicas. Estas formas se reproduzem por divisão binária e tornam-se ligadas às microvilosidades do epitélio do intestino médio. Esta adesão ocorre predominantemente em toda a região do flagelo e envolve a participação de um lipofosfoglicano exposto na superfície da promastigota (Pimenta et al., 1992). Quando os parasitos desprendem-se do epitélio, as formas promastigotas migram para a válvula estomodeu localizada no intestino médio anterior, onde se concentram e reiniciam a divisão celular (Gossage et al., 2003). Estes parasitos são responsáveis pela produção e secreção de um gel que atua como um tampão de obstrução do intestino médio e faringe do vetor (Rogers et al., 2002; Rogers, 2012). Durante esta etapa, os parasitos diferenciam-se em formas promastigotas metacíclicas infectantes, que estarão prontas para infectar um novo hospedeiro vertebrado (Fig. 4). É importante destacar que os parasitos pertencentes ao subgênero *Leishmania* desenvolvem-se no intestino anterior e médio do vetor, enquanto que os parasitos do subgênero *Viannia* desenvolvem-se no intestino posterior do flebotomíneo (Lainson et al., 1987).

1.3 Tratamento das leishmanioses

A ausência de vacinas de uso humano e programas eficazes de controle vetorial faz com que a quimioterapia seja uma das principais medidas utilizadas para controlar todas as formas da doença. Todavia, a alta toxicidade e os efeitos colaterais adversos dos fármacos utilizados no tratamento clínico, a possibilidade de surgimento de parasitos resistentes às drogas e a limitada opção de medicamentos são os principais desafios relacionados ao tratamento quimioterápico das leishmanioses (Murray, 2010).

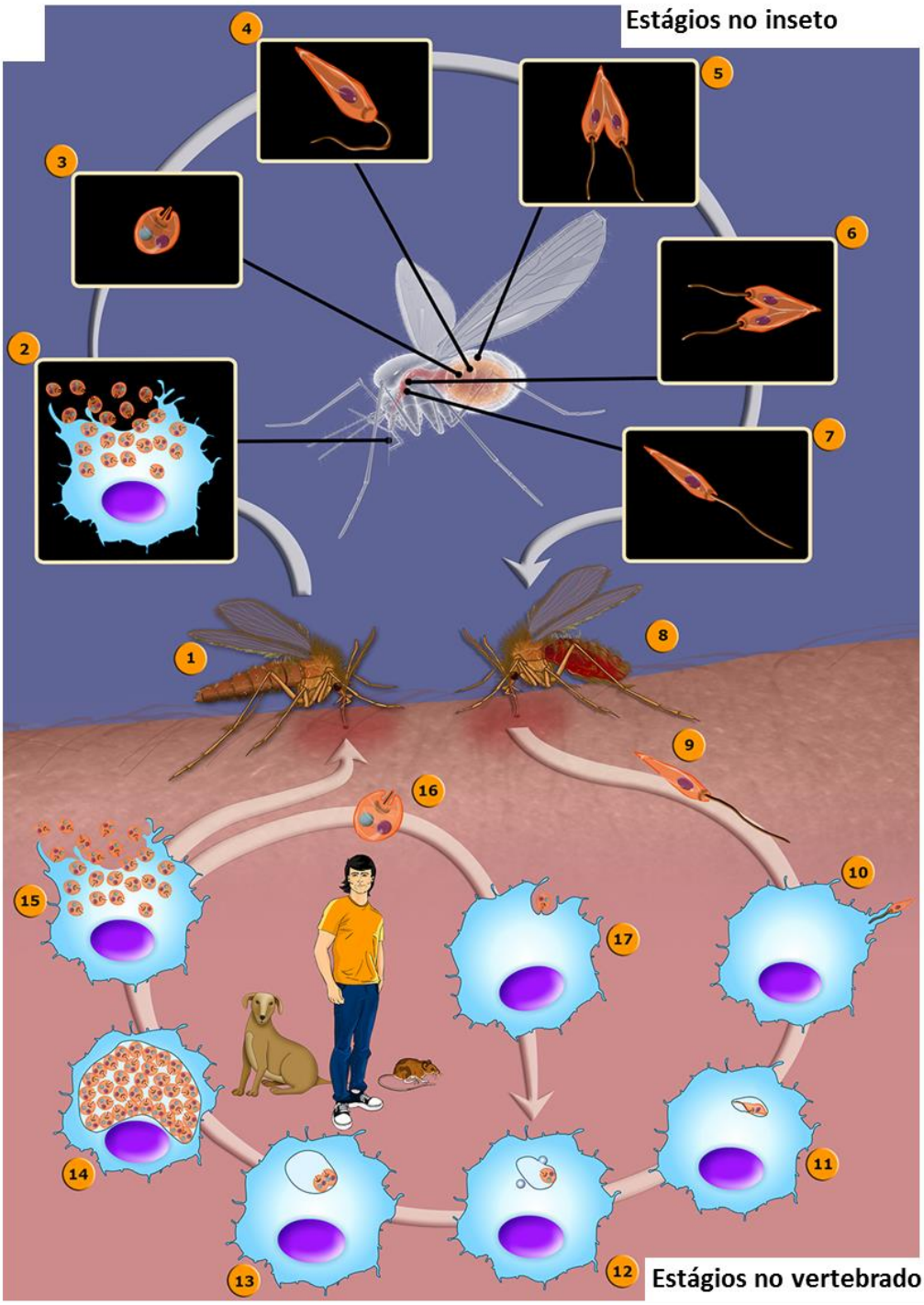

Figura 4 – Ciclo de vida de *Leishmania* spp. A fêmea de flebotomíneo pica um mamífero infectado durante o repasto sanguíneo (1). Macrófagos infectados com formas amastigotas (2). Forma amastigota (3). Amastigotas transformam-se em formas promastigotas procíclicas (4), que se multiplicam no intestino médio do vetor (5). Promastigotas migram em direção à válvula estomodeu no intestino médio anterior e a divisão celular reinicia (6). Promastigotas transformam-se em formas promastigotas metacíclicas infectantes (7). A fêmea libera estas formas em um novo hospedeiro vertebrado através da regurgitação durante o repasto sanguíneo (8). Promastigotas metacíclicos (9) infectam macrófagos (10). Promastigotas metacíclicos transformam-se em amastigotas (11). Amastigotas aderem-se à membrana do vacúolo parasitóforo (12) e multiplicam-se dentro dele (13). Multiplicação intensa de amastigotas (14), que são liberados da célula (15). Forma amastigota (16) que pode infectar novos macrófagos (17). Na porção central da figura, estão representados os reservatórios mais importantes envolvidos na manutenção do parasito (Adaptado de Teixeira et al., 2013).

No Brasil, o tratamento de primeira escolha para todas as formas da doença é a administração de compostos contendo o antimônio pentavalente (Sb^V). Os países da América Latina, língua francesa e espanhola comercializam o antimoniato de N-metilglucamina (Glucantime®), e os de língua inglesa, o estibogluconato de sódio (Pentostam®) (Berman, 1988; Rath, 2003). Apesar dos efeitos colaterais como arritmias cardíacas e problemas renais, estes fármacos têm sido utilizados por várias décadas em diversos países (Herwaldt, 1999). De acordo com o MS, no Brasil, as dosagens recomendadas para o tratamento da doença variam de 10 a 20 mg de Sb^V/kg/dia com aplicação por via parenteral, intramuscular ou endovenosa, por no mínimo 20 dias e no máximo 40 dias (Brasil, 2010, 2014). Entretanto, baixas dosagens ou descontinuidade do tratamento são as principais causas do aumento de recidivas e casos de resistência do parasito a essas drogas (Oliveira et al., 2011).

A anfotericina B e a pentamidina são os fármacos de segunda escolha utilizados no tratamento da doença em nosso país. A anfotericina B é um antibiótico poliênico com ação antifúngica, que foi introduzido na terapia anti-*Leishmania* a partir de 1960. Este fármaco liga-se aos esteróis levando à formação de poros na membrana e alteração na permeabilidade a íons, que por sua vez, promovem sua desorganização e despolarização, causando a morte do parasito (Ramos et al., 1994; Azas et al., 2001). Esse medicamento tem sido considerado o mais potente disponível comercialmente, com ação nas formas promastigotas e amastigotas do parasito, tanto *in vitro* quanto *in vivo* (Brasil, 2014). Em áreas onde há altos níveis de resistência ao Sb^V, como na Índia, a anfotericina B tem sido utilizada como primeira escolha no tratamento. Formulações lipídicas desse fármaco têm sido propostas como alternativa para diminuir a toxicidade do desoxicolato sódico de anfotericina B. Atualmente, as seguintes formulações tornaram-se disponíveis no mercado: anfotericina B lipossomal e anfotericina B dispersão coloidal (Brasil, 2014). Todavia, a anfotericina B lipossomal tem custo elevado, impossibilitando o seu uso na rotina dos serviços de saúde no Brasil. Ela tem sido indicada aos pacientes graves de LV, que desenvolveram insuficiência renal ou toxicidade cardíaca durante o uso do Glucantime® e de outras drogas que não resultaram em melhora ou cura clínica (Brasil, 2014).

A pentamidina, uma diamidina aromática, tem sido utilizada na quimioterapia das leishmanioses há mais de 40 anos. Os mecanismos antimicrobianos da pentamidina não são totalmente conhecidos, mas sabe-se que a droga interfere na

biossíntese de macromoléculas como DNA, RNA, fosfolipídeos e proteínas (Mcgwire & Satoskar, 2014). A considerável toxicidade desse fármaco e os principais efeitos adversos, como nefrotoxicidade, taquicardia e outras arritmias, hipoglicemia, dentre outros, tem restringido o seu uso (Singh et al., 2012).

A miltefosina oral, originalmente desenvolvida como um agente antineoplásico, apresenta atividade contra a membrana de *Leishmania* (Croft et al., 2006). Este medicamento tem apresentado resultados promissores no tratamento do calazar indiano (Brasil, 2014), sendo considerado como de primeira escolha na Índia e também na Colômbia. O mecanismo de ação da miltefosina não é conhecido com precisão, mas acredita-se que está associado à indução de apoptose e distúrbios nas vias de sinalização celular dependente de lipídeos (Dorlo et al., 2012). Esse fármaco parece ser mais ativo contra algumas espécies de *Leishmania*, entretanto sua eficácia é variável dependendo das áreas geográficas, inclusive para a mesma espécie (González et al., 2008). Morais-Teixeira et al. (2011) mostraram que miltefosina apresentou menor atividade em espécies de *Leishmania* do Novo Mundo quando comparado com *L. donovani*. Um estudo de genômica comparativa entre isolados de *L. chagasi* (*L. infantum*) demonstrou a deleção de um locus no cromossomo 31 (MSL – locus de sensibilidade à miltefosina), que estava fortemente associado com falha ao tratamento com miltefosina em pacientes com LV no Brasil (Carnielli et al., 2016).

A paromomicina, um antibiótico aminoglicosídeo, foi introduzido na quimioterapia das leishmanioses a partir de 2006, na forma de sulfato de paromomicina (Wiwanitkit, 2012). Este fármaco age na síntese de RNA e modifica lipídeos polares da membrana, afetando a sua fluidez e permeabilidade (Maarouf et al., 1997).

O Quadro 1 mostra os mecanismos de ação, o modo de administração e os efeitos colaterais dos principais fármacos utilizados no tratamento das leishmanioses. É importante ressaltar que a eficácia dessas drogas depende do estado imunológico do hospedeiro, das diferenças intrínsecas dos parasitos quanto à sensibilidade aos fármacos e das propriedades farmacocinéticas das drogas (Kaur & Rajput, 2014). A figura 5 mostra a estrutura química de alguns fármacos utilizados no tratamento da doença.

QUADRO 1 – Fármacos utilizados no tratamento das leishmanioses

Fármaco	Modo de ação	Modo de administração	Efeitos colaterais
Antimoniais pentavalentes	Inibição da glicólise e oxidação de ácidos graxos	Intramuscular ou Endovenosa	Dor abdominal, eritema, náuseas, toxicidade (hepática, pâncreas, renal, muscular, leucopenia)
Anfotericina B	Ligação aos esteróis da membrana do parasito e mudança na sua permeabilidade seletiva para K^+ e Mg^{2+}	Endovenosa	Febre, náuseas, anorexia, leucopenia, falha renal, problemas cardíacos
Pentamidina	Interfere na síntese de DNA e modifica a morfologia do cinetoplasto	Parenteral Intramuscular	Dores, náuseas, vômitos, tonturas, mialgia, hipertensão, dor de cabeça
Miltefosina	Associada com a biossíntese de fosfolipídeos e metabolismo alquil-lipídico em *Leishmania*	Oral	Náuseas, vômitos, diarreia, creatinina elevada
Paromomicina	Inibição da biossíntese de proteínas	Tópico para LC Parenteral para LV	Eritema, dores, edema, danos ao ouvido interno

Fonte: Revisado por Kaur & Rajput (2014).

1.4 Mecanismos de ação dos antimoniais

Antimoniais pentavalentes (Sb^V) são pró-drogas que necessitam de redução biológica para a forma trivalente (Sb^{III}) para terem atividade leishmanicida. Entretanto, o local desta redução, se dentro do parasito (forma amastigota) ou no macrófago, e o mecanismo de redução (enzimático ou não enzimático) permanecem com resultados controversos na literatura (Shaked-Mishan et al., 2001).

Alguns estudos têm relatado que amastigotas axênicos, cultivados na ausência de macrófagos, são susceptíveis ao Sb^V, enquanto em promastigotas este composto não é ativo, sugerindo que alguma redução ocorre nas formas

amastigotas do parasito (Callahan et al., 1997; Ephros et al., 1997; Ephros et al., 1999; Goyard et al., 2003). Por outro lado, outros estudos sugerem que a redução dessa pró-droga acontece somente no interior dos macrófagos (Roberts & Rainey, 1993; Sereno et al., 1998).

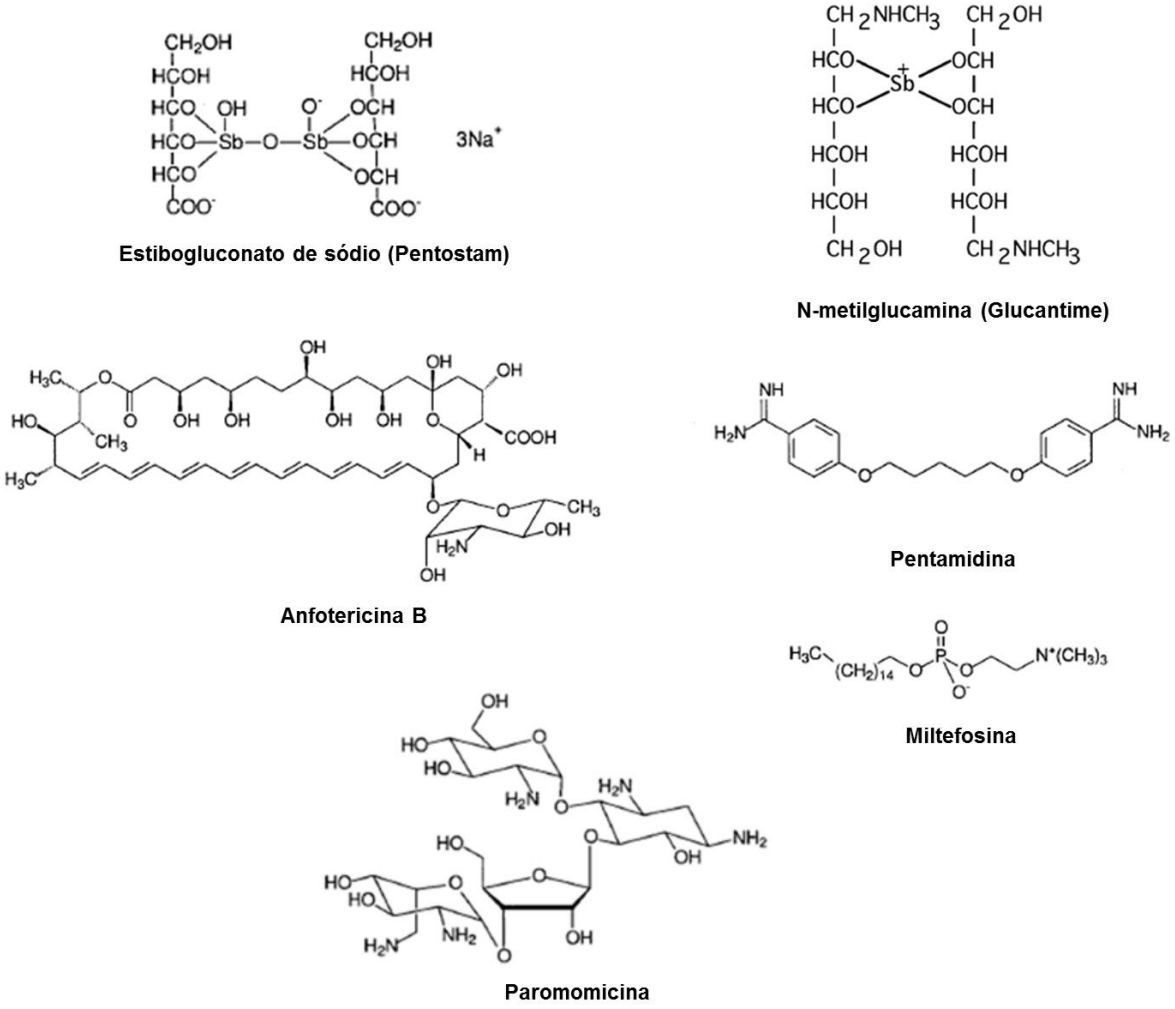

Figura 5 – Estrutura química dos fármacos utilizados no tratamento das leishmanioses (Adaptado de Croft et al., 2006; Santos et al., 2008).

O mecanismo pelo qual amastigotas reduzem Sb^{V} em Sb^{III} não é claro. Tanto glutationa quanto tripanotiona podem reduzir não enzimaticamente Sb^{V} a Sb^{III} sob condições ácidas (Ouellette et al., 1991; Frézard et al., 2001; Santos Ferreira et al., 2003; Yan et al., 2003a; Yan et al., 2003b). Entretanto, a relevância fisiológica dessas observações é uma questão a ser investigada devido ao fato das taxas de redução serem bastante lentas. Além disso, promastigotas contêm concentrações intracelulares maiores de tripanotiona e glutationa do que amastigotas (Ariyanayagam e Fairlamb, 2001; Wyllie et al., 2004) e ambos estágios mantêm valores intracelulares de pH próximos à neutralidade, independentemente do pH

externo (Glaser et al., 1988). Assim, é difícil explicar a ação seletiva de Sb^{V} contra o estágio amastigota por um mecanismo não enzimático. Como ambos os estágios podem absorver Sb^{V} e Sb^{III}, a insensibilidade de promastigotas ao Sb^{V} não pode ser atribuída à exclusão da droga (Brochu et al., 2003).

Sb^{III} é capaz de entrar em formas amastigotas e promastigotas de *Leishmania*, entretanto as rotas de entrada de Sb^{III} e Sb^{V} diferem (Brochu et al., 2003). Estudos mostram que Sb^{III} entra em *Leishmania* através de uma aquagliceroporina denominada AQP1 (Gourbal et al., 2004). Para ser ativo contra *Leishmania*, Sb^{V} tem que entrar na célula hospedeira, atravessar a membrana fagolisossomal e agir contra as formas amastigotas intracelulares (Shaked-Mishan et al., 2001). Acredita-se que Sb^{V} entra no parasito através de uma proteína que reconhece uma estrutura semelhante a um açúcar compartilhado com gluconato (Brochu et al., 2003).

É possível que a redução de Sb^{V} a Sb^{III} ocorra tanto no hospedeiro quanto no parasito. Em bactéria e levedura, a redução de metal é mediada por enzimas (Rosen, 2002) e isso pode também acontecer em *Leishmania*. Uma enzima específica do parasito denominada redutase dependente de tiol 1 (TDR1), que contém domínios com similaridades à ômega glutationa transferase, mostrou-se catalisar a conversão de Sb^{V} a Sb^{III}, usando glutationa como um agente redutor (Denton et al., 2004). Uma nova enzima antimoniato redutase 2 (ACR2) foi caracterizada em *Leishmania* e mostrou-se reduzir Sb^{V} e aumentar a sensibilidade de células de *Leishmania* ao Sb^{V} (Zhou et al., 2004). Alternativamente, há evidência que um número de tióis, incluindo tióis parasito-específicos tais como tripanotiona, assim como tióis macrófagos-específicos tais como glicilcisteína, podem reduzir Sb^{V} a Sb^{III}, não enzimaticamente (Fig. 6) (Santos Ferreira et al., 2003).

Estudos sugerem que o Sb^{V} inibe a biossíntese de macromoléculas em amastigotas através de perturbação do metabolismo energético, devido à inibição da atividade glicolítica, oxidação de ácidos graxos e a fosforilação de ADP para a formação de ATP (Berman et al., 1985; Berman et al., 1987; Herwaldt, 1999). Entretanto, os alvos específicos nestas vias não estão bem identificados. Outros estudos têm relatado apoptose em amastigotas tratados com Sb^{III}, envolvendo fragmentação de DNA e exteriorização de fosfatidilserina na superfície externa da membrana plasmática (Sereno et al., 2001; Sudhandiran & Shaha, 2003). Dados da literatura sugerem a formação de complexos aquosos entre o Sb^{V} e

ribonucleosídeos, adenosina e adenosina monofosfato em compartimentos celulares ácidos, tais como o fagolisossomo do macrófago, interferindo no metabolismo da *Leishmania* e podendo causar a morte do parasito (Demicheli et al., 2002). Além disso, estudos sugerem que Sb^{III} causa distúrbios no potencial tiol redox do parasito, através da ativação do efluxo de tripanotiona e glutationa, com a produção de espécies reativas de oxigênio (EROs), o que contribuiria para a morte do parasito, uma vez que este se tornaria susceptível ao estresse oxidativo (Wyllie et al., 2004; Ashutosh et al., 2007).

1.5 Mecanismos de resistência aos antimoniais

A resistência a drogas, especialmente aos antimoniais, é um grave problema associado à quimioterapia das leishmanioses em vários países do mundo. Na Índia, mais de 60% dos pacientes, os quais não foram tratados previamente, falharam ao responder à terapia com antimoniais, devido à resistência natural (Sundar, 2001; Thakur et al., 2004). Dessa maneira, o uso de antimoniais não têm sido recomendado no tratamento das leishmanioses no subcontinente indiano (Guerin et al., 2002; Matlashewski et al., 2011). Uma possível explicação para o surgimento de resistência nesta região deve-se ao uso de arsênio na água potável (Perry et al., 2011). Falhas no tratamento com antimoniais também têm sido relatadas no Brasil (Oliveira Neto et al., 1997; Romero et al., 2001), Colômbia (Palacios et al., 2001) e Peru (Arevalo et al., 2007). No Brasil, a ineficácia do tratamento com Glucantime® tem sido observada em 10 a 25% dos pacientes tratados (Rocha et al., 1980; Marsden et al., 1984).

O fenômeno de resistência de *Leishmania* aos antimoniais é complexo, multifatorial e envolve diversas vias. Dessa maneira, muitos estudos têm sido desenvolvidos para compreender melhor a bioquímica deste parasito e subsidiar o entendimento dos possíveis mecanismos pelos quais o parasito adquire resistência. Dados da literatura demonstram que os potenciais mecanismos de resistência aos antimoniais em espécies de *Leishmania* incluem: (1) diminuição da absorção da droga pela célula; (2) diminuição da ativação da droga; (3) inativação da droga; (4) alteração do complexo alvo-droga; (5) sequestro da droga em compartimentos intracelulares; (6) aumento do reparo de danos causados pela droga; (7) aumento da

tolerância à droga e (8) aumento da extrusão da droga (Fig. 6) (Borst, 1991; Croft et al., 2006; Singh, 2006).

Estudos experimentais de resistência ao antimônio em *Leishmania* indicam que vários mecanismos devem ocorrer simultaneamente no mesmo parasito e que diferentes mecanismos devem operar em isolados de campo comparados a linhagens resistentes de *Leishmania* selecionadas em laboratório (Ouellette et al., 2004; Croft et al., 2006; Decuypere et al., 2012). Por outro lado, algumas características observadas em estudos *in vitro* também têm sido demonstradas em isolados de campo (Decuypere et al., 2005; Mukherjee et al., 2007; Kumar et al., 2012; Berg et al., 2013; Kazemi-Rad et al., 2013; Rai et al., 2013). O Quadro 2 apresenta alguns genes frequentemente associados com o fenótipo de resistência ao antimônio em diferentes mutantes de *Leishmania* spp. selecionados *in vitro* em laboratório ou em isolados clínicos e/ou de campo.

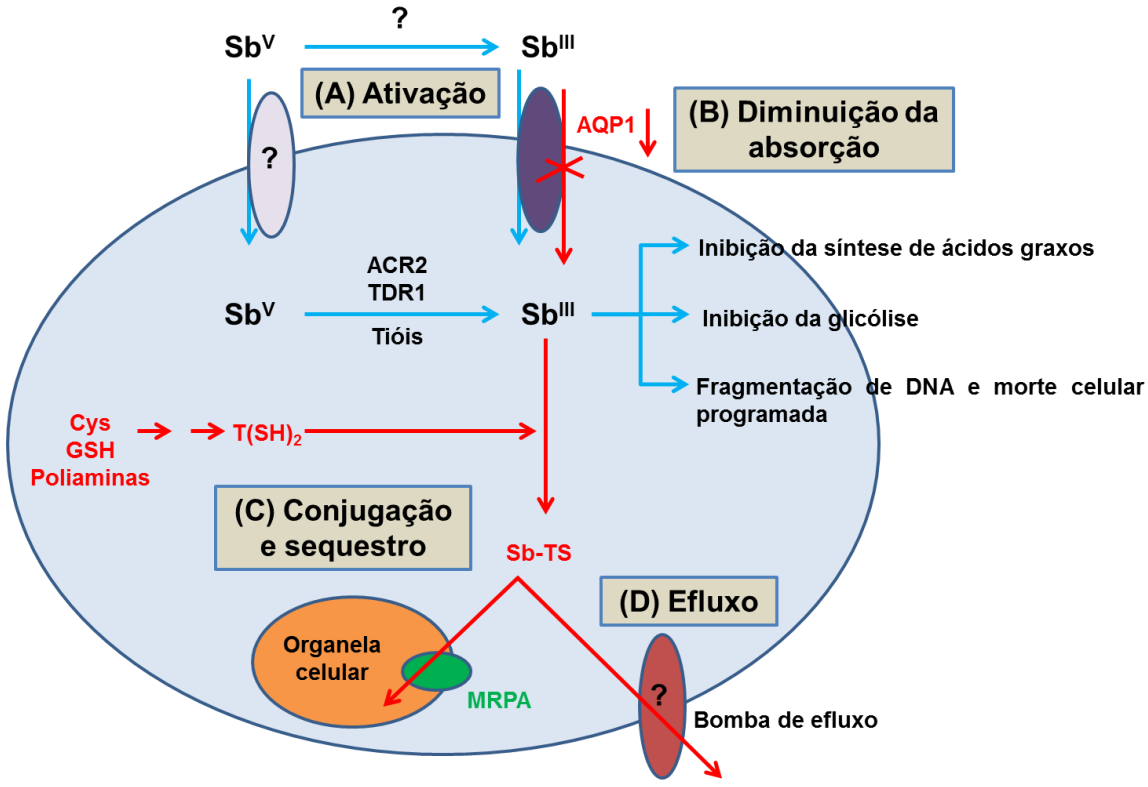

Figura 6 – Mecanismos de ação e resistência aos antimoniais em *Leishmania*. Ativação (A), diminuição da absorção (B), conjugação e sequestro (C), e efluxo (D). As setas azuis indicam a provável ação da droga em linhagens sensíveis de *Leishmania*, enquanto as setas vermelhas descrevem as prováveis rotas para obter a resistência, como observado nas linhagens resistentes do parasito. Abreviaturas: Sb^{V} (antimônio pentavalente); Sb^{III} (antimônio trivalente); AQP1 (aquagliceroporina 1); ACR2 (antimoniato redutase 2); TDR1 (redutase dependente de tiol 1); Cys (cisteína); GSH (glutationa); $T(SH)_2$ (tripanotiona); Sb-TS (complexo antimônio-tiol); MRPA (proteína de resistência a múltiplas drogas A) (Adaptado de Kaur & Rajput, 2014).

QUADRO 2 – Genes associados com o fenótipo de resistência ao antimônio em mutantes de *Leishmania* spp. selecionados *in vitro* ou em isolados clínicos/campo

Gene	***Leishmania* spp.**	**Resistência ao antimônio**		**Referência**
		In vitro	**Isolado clínico/campo**	
MRPA	*L. guyanensis*		X	Anacleto et al. (2003)
	L. infantum	X		El Fadili et al. (2005) Leprohon et al. (2009)
	L. donovani		X	Mukherjee et al. (2007) Kumar et al. (2012) Rai et al. (2013)
	L. amazonensis	X		do Monte Neto et al. (2011) Moreira et al. (2013)
	L. tropica		X	Kazemi-Rad et al. (2013)
	L. braziliensis	X		Moreira et al. (2013)
AQP1	*L. donovani*		X	Decuypere et al. (2005) Kumar et al. (2012)
	L. tropica		X	Kazemi-Rad et al. (2013)
	L. donovani	X	X	Rai et al. (2013)
	L. amazonensis *L. guyanensis*	X		Moreira et al. (2013)
ODC	*L. guyanensis*	X		Fonseca et al. (2017)
	L. donovani		X	Decuypere et al. (2005) Mukherjee et al. (2007) Adaui et al. (2011) Rai et al. (2013)
γ-GCS	*L. guyanensis*	X	X	Torres et al. (2010) Fonseca et al. (2017)
	L. donovani		X	Decuypere et al. (2005) Mukherjee et al. (2007) Kumar et al. (2012) Rai et al. (2013)
TXNPx	*L. donovani*		X	Wyllie et al. (2010)
	L. braziliensis	X		Matrangolo et al. (2013) Andrade & Murta (2014)
FeSOD-A	*L. braziliensis* *L. infantum*	X		Tessarollo et al. (2014)

Fonte: Adaptado de Frézard et al. (2014).
Abreviaturas: *MRPA* (proteína de resistência a múltiplas drogas A); *AQP1* (aquagliceroporina 1); *ODC* (ornitina decarboxilase); *γ-GCS* (γ-glutamilcisteína sintetase); *TXNPx* (triparedoxina peroxidase); *FeSOD-A* (ferro superóxido dismutase-A); X indica resistência *in vitro* ou em isolado clínico/campo.

É evidente que o comprometimento da redução de Sb^{V} a Sb^{III} é um dos primeiros meios de resistência a drogas em *Leishmania* (Fig. 6A). Uma diminuição dessa redução foi demonstrada em *L. donovani* resistente ao antimônio (Shaked-Mishan et al., 2001). Está bem estabelecido que o transporte de Sb^{III} para o interior do parasito ocorre através de um transportador de membrana denominado aquagliceroporina 1 (AQP1), de maneira independente de energia (Brochu et al., 2003; Gourbal et al., 2004). Os níveis intracelulares de Sb^{III} estavam reduzidos em isolados clínicos de *L. major*, *L. infantum* e *L. tarentolae* resistentes, provavelmente devido a uma baixa expressão de AQP1, demonstrando seu papel significativo na resistência aos antimoniais (Fig. 6B) (Gourbal et al., 2004). Outros estudos evidenciaram que a reduzida expressão (Marquis et al., 2005) ou a deleção do gene *AQP1* (Mukherjee et al., 2013) deve resultar na diminuição da entrada de Sb^{III}, reduzido acúmulo da droga e resistência. De fato, dados da literatura revelaram uma diminuição da expressão dessa proteína em isolados de campo de *L. tropica* e *L. donovani* (Decuypere et al., 2005; Kumar et al., 2012; Kazemi-Rad et al., 2013; Rai et al., 2013), e em linhagens de *L. guyanensis* e *L. amazonensis* resistentes *in vitro* ao Sb^{III} (Moreira et al., 2013), provocando uma redução da entrada da droga na célula parasitária.

O aumento dos níveis de tripanotiona foi observado em algumas linhagens de *Leishmania* selecionadas para resistência ao Sb^{III} ou arsenito (Mukhopadhyay et al., 1996). Isso é devido ao aumento dos níveis de enzimas envolvidas na síntese de glutationa (γ-glutamilcisteína sintetase) (Grondin et al., 1997) e poliaminas (ornitina decarboxilase) (Haimeur et al., 1999), os dois metabólitos precursores da tripanotiona. Aumento da síntese de glutationa e tripanotiona pode ajudar a substituir tióis perdidos devido ao efluxo, bem como auxiliar no restabelecimento do potencial tiol redox alterado pelo acúmulo de dissulfetos (Wyllie et al., 2004).

Uma vez que o Sb^{III} está no interior do parasito, ele pode formar conjugados com os tióis tripanotiona ($T(SH)_2$), glutationa (GSH) e cisteína (Cys), resultando na formação do complexo antimônio-tiol (Sb-TS). Dessa maneira, outro mecanismo associado à resistência aos antimoniais é o destino deste complexo através de duas principais vias: sequestro em compartimento intracelular do parasito por meio de um transportador ABC (*ATP-binding cassette*) e eliminação da célula através da membrana plasmática (Fig. 6C e D) (Mukhopadhyay et al., 1996; Légaré et al., 2001). O transportador ABCC3, também conhecido como PGPA

(*phosphoglycoprotein* A) ou MRPA (*multidrug resistant associated protein* A), foi identificado no sequestro do complexo Sb-tiol em uma organela intracelular de *Leishmania*, levando à remoção de moléculas da droga do citoplasma do parasito (El Fadili et al., 2005). A associação do aumento da expressão da proteína PGP na resistência a drogas foi descrita em *Leishmania* resistente ao arsenito e antimoniais (Légaré et al., 2001), sugerindo que este polipeptídeo seja um dos principais mediadores do efluxo de drogas neste parasito. Um estudo demonstrou que a PGP está aumentada em linhagens de *L. guyanensis* e *L. amazonensis* resistentes *in vitro* ao Sb^{III}, podendo levar à uma redução na concentração intracelular de antimônio e favorecer a sobrevivência destes parasitos resistentes (Moreira et al., 2013). Manzano et al. (2013) identificaram que o transportador ABCI4 também está envolvido no efluxo do conjugado Sb-tiol em células de *L. major* resistentes ao antimonial.

Após a infecção, macrófagos sofrem uma explosão respiratória, produzindo espécies reativas de oxigênio (EROs) e nitrogênio (ERNs), como H_2O_2 (peróxido de hidrogênio), OH^- (radical hidroxila), O_2^- (ânion superóxido) e NO (óxido nítrico) para destruir microrganismos invasores (Beaman & Beaman, 1984; Bhattacharyya et al., 2002). Dessa forma, muitos parasitos, incluindo *Leishmania*, desenvolveram diferentes mecanismos enzimáticos para prevenir o dano celular causado por tais EROs e ERNs. A função de peroxidases inclui a defesa contra agentes químicos e estresse oxidativo, catalizando a redução de H_2O_2 e hidroperóxidos a água e álcool, respectivamente. Sendo assim, a ação combinada de tripanotiona redutase, triparedoxina e triparedoxina peroxidase é crucial para manter uma baixa concentração de H_2O_2 e diminuir o estresse oxidativo (Turrens, 2004). Estudo proteômico demonstrou que triparedoxina peroxidase citosólica está superexpressa em linhagens de *L. braziliensis* e *L. infantum* resistentes ao Sb^{III} (Matrangolo et al., 2013). Análise funcional desta proteína sugere que ela está envolvida no fenótipo de resistência ao antimônio trivalente em *L. braziliensis* (Andrade & Murta, 2014). A enzima superóxido dismutase (SOD) também é um componente central envolvido na defesa antioxidante em muitos microrganismos. Esta enzima remove o excesso de radicais O_2^-, convertendo-os em oxigênio e H_2O_2 (Bannister et al., 1987). Diferentes isoformas de FeSOD (FeSOD-A e FeSOD-B) têm sido caracterizadas em *L. chagasi*, *L. tropica* e *L. donovani* (Paramchuk et al., 1997; Ghosh et al., 2003; Plewes et al., 2003; Getachew & Gedamu, 2007), demonstrando seu papel protetor contra o

estresse oxidativo. Estudo de análise funcional de FeSOD-A revelou que a atividade SOD é maior nas linhagens de *L. braziliensis* e *L. infantum* resistentes ao Sb^{III} comparadas com suas respectivas linhagens sensíveis, sugerindo que esta enzima esteja envolvida no fenótipo de resistência ao Sb^{III} nestas duas espécies de *Leishmania* (Tessarollo et al., 2014).

Estudos genômicos têm demonstrado que a amplificação gênica é um dos principais mecanismos envolvidos na resistência a drogas, principalmente quando a resistência é induzida *in vitro* (Beverley, 1991; Segovia, 1994; Borst & Ouellette, 1995). Amplificação gênica associada com o fenótipo de resistência ao antimonial normalmente ocorre por recombinação homóloga de sequências repetidas diretas ou invertidas no genoma (Beverley, 1991; Ouellette & Borst, 1991; Grondin et al., 1996; Leprohon et al., 2009), levando à formação de elementos de DNA extracromossomais circulares ou lineares (Mukherjee et al., 2007; Leprohon et al., 2009; do Monte-Neto et al., 2011). A plasticidade cromossomal em *Leishmania* permite mudanças estruturais, resultando em variações no número de cópias cromossômicas e amplificação de DNA extracromossomal (Downing et al., 2011). De fato, aneuploidia é um fenômeno que tem sido demonstrado em *Leishmania* resistente ao antimonial (Ubeda et al., 2008; Leprohon et al., 2009). Sendo assim, o aumento do número de cópias gênicas devido à amplificação extracromossomal e/ou intracromossomal deve contribuir para a superexpressão de genes, resultando em adaptações metabólicas que auxiliam os parasitos a sobreviver na presença de antimônio (Frézard et al., 2014).

Novos mecanismos de resistência ao antimonial em *Leishmania* têm sido identificados, como diminuição na fragmentação de DNA e morte celular programada (Vergnes et al., 2007). Estudos de metaboloma revelaram uma diminuição dos níveis de esfingolipídeos e esfingomielinas, e aumento dos níveis de fosfatidilcolina e ácidos graxos insaturados em isolados clínicos de *L. donovani* resistentes ao antimônio, sugerindo que a composição da membrana destes parasitos esteja extensivamente modificada (t'Kindt et al., 2010). Níveis de glicoconjugados de superfície também foram encontrados aumentados em isolados de *L. donovani* resistentes ao antimônio, desempenhando papel na metaciclogênese e virulência do parasito (Mukhopadhyay et al., 2011). Além disso, acredita-se que uma diminuição na expressão de calcineurina esteja associada com resistência ao Glucantime® em

isolados naturais de *L. infantum*, devido a um efeito protetor dos parasitos resistentes à apoptose induzida pelo antimônio (Bagher Khadem Erfan et al., 2013).

Portanto, o surgimento de amostras de *Leishmania* resistentes à droga e o aumento da propagação de espécies resistentes deste parasito enfatizam a importância de identificação de mecanismos diretamente envolvidos na resistência à droga, assim como as mudanças fisiológicas que podem ocorrer nestes parasitos. Tais mudanças fisiológicas, relacionadas ou não a mecanismos de resistência à droga, contribuem para as características globais do fenótipo de resistência. Além disso, novas abordagens quimioterapêuticas contra leishmanioses poderiam ser desenvolvidas a partir desses estudos de resistência (Ponte-Sucre, 2003).

1.6 Regulação da expressão gênica em tripanossomatídeos

A regulação da expressão gênica em tripanossomatídeos apresenta as seguintes características peculiares: transcrição policistrônica, *transplicing*, transcrição por RNA polimerase I de alguns genes codificadores de proteínas, ausência de regiões promotoras típicas para a transcrição dos genes codificadores de proteína pela RNA polimerase II, cromatina menos condensada, edição de RNAs mitocondriais e disposição dos genes *em tandem* (Clayton, 2002; Campbell et al., 2003). O genoma destes organismos não apresenta *introns,* de forma que o DNA genômico pode ser utilizado como molde para realização da reação em cadeia da polimerase (PCR). Além do DNA nuclear, parasitos da família Trypanosomatidae apresentam o cinetoplasto, que consiste no DNA mitocondrial ou DNA do cinetoplasto (kDNA), presente na mitocôndria única desses parasitos. Este DNA mitocondrial é formado por maxicírculos e minicírculos que se unem para formar uma rede de DNA, que representa 10-15% do DNA total do parasito. Nos maxicírculos, encontra-se o DNA que codifica RNAs ribossomais e transportadores, enquanto no DNA dos minicírculos localizam-se os RNAs guias (Simpson, 1987; Shapiro & Englund, 1995; Morris et al., 2001).

Os tripanossomatídeos apresentam transcrição policistrônica, gerando RNAs imaturos que contêm mais de um gene, como ocorre em procariotos (González et al., 1985; Muhich & Boothroyd, 1988). A maturação do mRNA ocorre através de um processo denominado de *transplicing*, de modo que na região 5’ dos RNAs codificantes é adicionada uma sequência de 39 nucleotídeos denominada miniexon

ou *spliced leader* (SL), e na região 3’ uma cauda poli-A, mediante um mecanismo em que ambos processos parecem estar acoplados (Borst, 1986; LeBowitz et al., 1993). É importante ressaltar que sinais da região intergênica, rica em resíduos de polipirimidinas (CT – citosina e timina) essenciais no controle da transcrição, determinam qual sítio AG (adenina e guanina) seja um possível receptor do SL (Martínez-Calvillo et al., 2010). Além disso, a região 3’ de cada mRNA está poliadenilada a uma distância entre 100 e 400 nucleotídeos acima dos sinais para o *transplicing* do miniexon do gene seguinte (Papadopoulou et al., 2003). Dessa maneira, formam-se unidades de tradução monocistrônicas compostas pelo miniexon, gene e cauda poli-A. Essa estrutura *cap* ou miniexon protege o mRNA da ação de nucleases, prolongando a sua vida média e assegurando a tradução, porque faz do mRNA uma estrutura reconhecida pelos ribossomos (Papadopoulou et al., 2003).

Uma vez que todos os genes são transcritos igualitariamente no agrupamento policistrônico, a regulação pós-transcricional é essencial para a manutenção natural dos níveis de transcritos. Alguns elementos regulatórios também foram descritos em vários mRNAs, sugerindo um mecanismo para a manutenção da estabilidade e degradação de mRNAs originalmente transcritos nos agrupamentos policistrônicos. Isso pode explicar a não correlação de níveis de mRNA com o nível proteico, sugerindo que a regulação da expressão gênica em tripanossomatídeos seja essencialmente pós-transcricional (Martínez-Calvillo et al., 2010). Desse modo, a regulação pode ocorrer nos seguintes pontos: processamento do transcrito primário, exportação do núcleo para o citoplasma, estabilidade do mRNA, recrutamento de RNA para polissomos, controle do início da tradução, modificações pós-traducionais e degradação de proteínas (Fig. 7).

Outra característica peculiar dos tripanossomatídeos é a ausência visível de condensação cromossômica em todo o seu ciclo de vida (Vickerman & Preston, 1970). Estes organismos caracterizam-se por sua grande plasticidade cromossômica, apresentando diploidia para a maioria dos genes e aneuploidia para outros (Lighthall & Giannini, 1992). Além disso, a edição de RNA mitocondrial ou RNA *editing* consiste na inserção e eliminação de uracila em RNAs sem sentido genético com a finalidade de obter mensageiros que serão traduzidos (Stuart & Panigrahi, 2002). Um complexo de moléculas de RNA e proteínas auxiliado pelos RNAs guias está envolvido no mecanismo de transcrição. Dessa maneira, foram

isolados numerosos fatores da mitocôndria de tripanossomatídeos, formando complexos que interagem dinamicamente durante o processo de edição (Simpson et al., 2003).

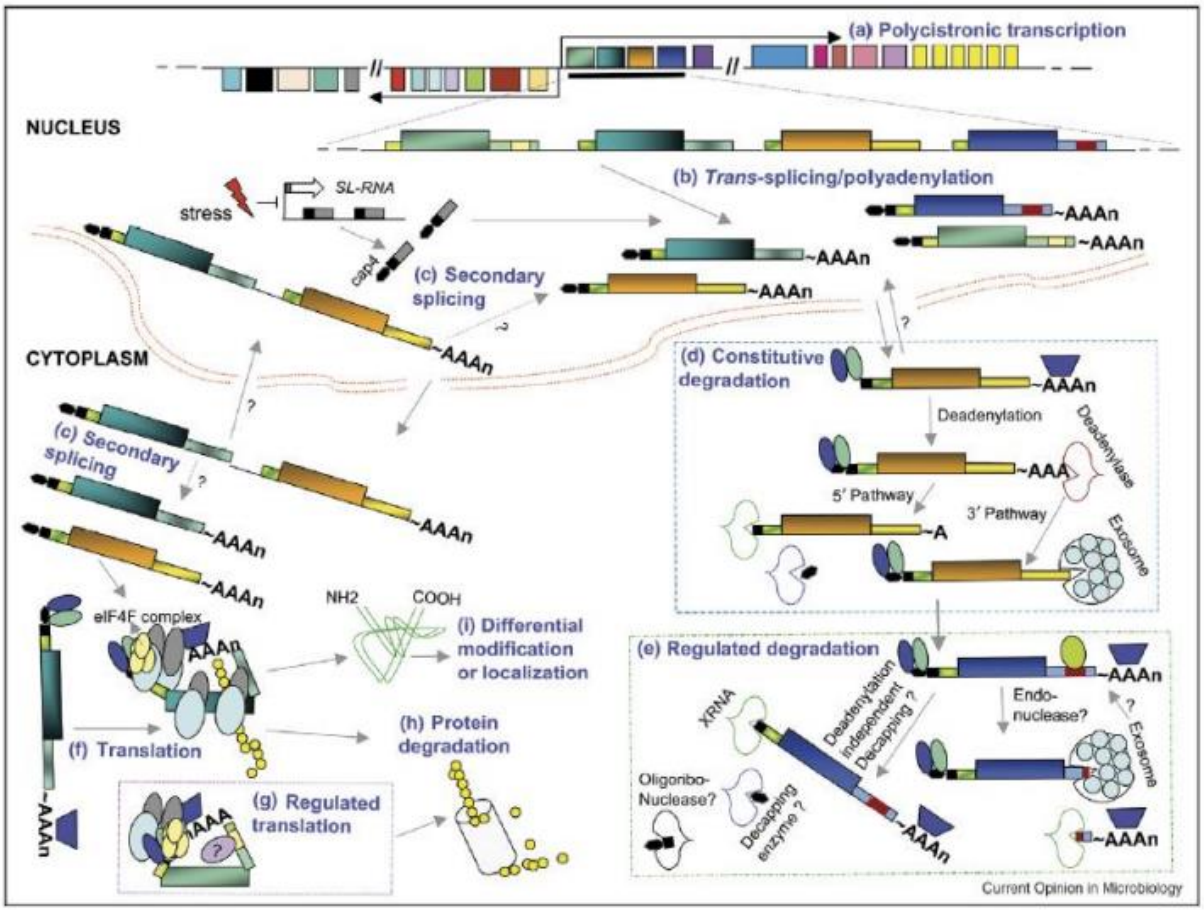

Figura 7 – Mecanismos de controle da expressão gênica em tripanossomatídeos. Transcrição policistrônica (A). *Transplicing* e poliadenilação acoplados durante a transcrição (B). Processamento de mRNAs dicistrônicos que são armazenados e, posteriormente, são novamente processados gerando mRNAs monocistrônicos (C). Degradação do mRNA dependente de desadenilação pela extremidade 5' e/ou 3' (D). Degradação do mRNA independente de desadenilação (E). Início da tradução (F). Alongamento da tradução (G). Degradação de proteínas (H). Modificações pós-traducionais de proteínas ou direcionamento da localização da proteína (I). (?) indica questões não confirmadas experimentalmente (Haile & Papadopoulou, 2007).

1.7 Organização genômica de *Leishmania*

O sequenciamento dos genomas de três protozoários da Ordem Kinetoplastida (*L. major*, *Trypanosoma brucei* e *T. cruzi*) mostrou alta conservação dos genes na evolução destes parasitos. Há cerca de 6.200 genes conservados na família Trypanosomatidae e mais de 1.000 genes específicos de *Leishmania*, muitos dos quais ainda não foram caracterizados (Peacock et al., 2007).

Apesar de uma divergência de aproximadamente 46 milhões de anos, a análise comparativa do genoma de três espécies de *Leishmania*, *L. major* (cepa Friedlin), *L. infantum* (cepa JPCM5) e *L. braziliensis* (cepa M2904), mostrou um alto grau de conservação dos genes e pequeno número de genes espécie-específicos destes parasitos (Ivens et al., 2005; Lukes et al., 2007; Peacock et al., 2007). A análise comparativa destes três genomas (Peacock et al., 2007) mostrou que aproximadamente 200 genes (pseudogenes) são diferentes, incluindo 78 genes que foram restritos a uma única espécie. Segundo os autores, o pequeno número de genes diferentes entre as três espécies pode ser compensado por um maior controle pós-transcricional (Peacock et al., 2007). A maioria desses genes espécie-específicos codifica proteínas de funções desconhecidas e tem sido proposto que contribuem para o tropismo do parasito e estejam associados com as diferentes formas de leishmanioses (Peacock et al., 2007; Smith et al., 2007). Vários genes específicos de *L. donovani* foram expressos em *L. major* e mostraram aumento significativo da sobrevivência do parasito em vísceras de camundongos, indicando que genes individuais podem contribuir para o tropismo no hospedeiro (Zhang et al., 2008; Zhang & Matlashewski, 2010). Dessa maneira, foi observada a conservação em mais de 99% dos genes preditos nesses genomas; as sequências de aminoácidos têm em média 77 a 92% de identidade, e as sequências de nucleotídeos, 81 a 94%.

O número de cromossomos é de 36 em *L. major* e *L. infantum*, e 35 em *L. braziliensis*, como resultado da fusão dos cromossomos 20 e 34 (Britto et al., 1998; Peacock et al., 2007). O genoma de *L. mexicana* apresenta 34 cromossomos, com dois eventos de fusão ocorridos entre os cromossomos 8 e 29, e entre os cromossomos 20 e 36 (Britto et al., 1998). Rogers et al. (2011) encontraram dois genes únicos presentes no genoma de *L. mexicana* (cepa U1103), os quais codificam proteínas de função desconhecida. Além disso, estes autores relataram a presença de grupos ortólogos desta espécie mais em comum com *L. infantum* do que qualquer outra espécie de *Leishmania*. Raymond et al. (2012) encontraram um limitado número de regiões cromossômicas divergentes entre as espécies *L. tarentolae* e *L. infatum* e um compartilhamento do conteúdo gênico maior que 90% de *L. tarentolae* com outras espécies de *Leishmania*. Estes autores também identificaram 95 sequências codificantes que são únicas em *L. tarentolae* e 250 genes que estavam ausentes nesta espécie. Dessa forma, vários dos genes que não

estão presentes em *L. tarentolae* são expressos preferencialmente nas formas amastigotas das espécies patogênicas de *Leishmania*. Isso poderia explicar, em parte, porque *L. tarentolae* é menos adaptada a infectar macrófagos humanos, e porque geralmente esta espécie de parasito não é patogênica para lagartos.

1.8 Análise proteômica e resistência a drogas

O termo proteoma foi utilizado pela primeira vez em 1995 para designar a análise extensiva das proteínas expressas em um tecido, célula ou compartimento subcelular em determinadas condições (Wilkins et al., 1996). Enquanto o genoma de um organismo permanece relativamente estável ao longo da sua vida, o proteoma é extremamente dinâmico e variável, podendo ser modificado pelas condições e estímulos em que esse organismo está exposto. A análise proteômica não inclui somente a identificação e quantificação de proteínas, mas também determina sua localização, interações, atividades, funções e modificações (Fields, 2001). De forma geral, o objetivo bioquímico do proteoma é o produto gênico responsável pelo fenótipo (Cuervo et al., 2010).

A pesquisa proteômica tem permitido o entendimento de processos metabólicos e fisiológicos, bem como a identificação e caracterização de marcadores biológicos, visando o diagnóstico precoce de doenças e acompanhamento da evolução do tratamento. Além disso, pode viabilizar também a identificação de novas moléculas bioativas que podem ser utilizadas para o desenvolvimento de novos fármacos para o controle de doenças e apontar novos candidatos vacinais e alvos terapêuticos.

Uma vez que a regulação da expressão gênica em tripanossomatídeos ocorre em níveis pós-transcricionais, abordagens proteômicas têm sido empregadas para detectar alterações de expressão de proteínas em espécies de *Leishmania* (Clayton & Shapira, 2007; Cuervo et al., 2010; Zilberstein, 2015). De fato, estudos anteriores utilizaram perfis proteômicos de espécies de *Leishmania* do Velho e do Novo Mundo para investigar fatores de virulência, proteínas estágio-específico, potenciais alvos de drogas e mecanismos de resistência a drogas (Bente et al., 2003; Drummelsmith et al., 2003; Walker et al., 2006; Vergnes et al., 2007; Matrangolo et al., 2013).

O primeiro estudo proteômico com o objetivo de compreender mecanismos de resistência a drogas em *Leishmania* foi relatado por Drummelsmith et al. (2003).

Inicialmente, estes autores validaram o uso de eletroforese bidimensional (2DE) para este tipo de estudo. O principal experimento desse trabalho foi a indução de resistência *in vitro* ao metotrexato em promastigotas de *L. major*. Análises proteômicas de 2DE comparativas mostraram que os parasitos resistentes apresentaram maior expressão de pteridina redutase (PTR1) comparado aos seus pares sensíveis nesta espécie do parasito. Sendo assim, os autores revelaram ferramentas valiosas para o estudo de potenciais alvos de drogas e mecanismos de resistência a drogas.

Vergnes et al. (2007) conduziram análise comparativa de cepas de *L. donovani* sensível e resistente ao Sb^{V} isoladas de pacientes com calazar. Resultados deste estudo revelaram que HSP83 e calpaína estão envolvidas com morte celular programada induzida pelo antimônio.

Estudo proteômico comparativo de formas amastigotas axênicas de *L. infantum* sensíveis e resistentes ao Sb^{III} demonstrou que a expressão da proteína argininosuccinato sintetase (ARGG) estava aumentada no mutante resistente à droga. Por outro lado, foi observada uma diminuição no nível da proteína de membrana dos kinetoplastídeos-11 (KMP-11) nestes parasitos resistentes (El Fadili et al., 2009).

Kumar et al. (2010) identificaram proteínas diferencialmente expressas em frações enriquecidas de membrana e citosólica em isolados clínicos de *L. donovani* sensível e resistente ao estibogluconato de sódio de pacientes com LV. Estes autores observaram que as principais proteínas na fração de membrana foram: transportador ABC, HSP83, proteína transamidase GPI, proteína rica em cisteína-leucina e proteína L23a ribossomal 60S, sugerindo que estas proteínas de membrana são importantes na transdução de sinais e na resistência à droga. Na fração citosólica, foram identificadas as seguintes proteínas: antígeno nuclear de proliferação celular (PCNA), subunidade alfa 5 do proteassoma, carboxipeptidase, HSP70, enolase, frutose-1,6-bifosfato aldolase, e a cadeia beta-tubulina. Muitas dessas proteínas têm sido relatadas como potenciais alvos de drogas, exceto a L23a ribossomal 60S e PCNA. Sendo assim, as proteínas descritas nesse estudo fornecem um vasto campo a ser explorado para novas estratégias de tratamento contra a LV, como a clonagem e superexpressão desses alvos para produzir proteínas recombinantes terapêuticas/profiláticas.

Biyani et al. (2011) utilizaram espectrometria de massas quantitativa para identificar diferenças no proteoma global entre os isolados de *L. donovani* sensíveis e resistentes ao estibogluconato de sódio. Dados das análises comparativas revelaram que nos parasitos resistentes à droga houve alterações de expressão de proteínas envolvidas em importantes vias metabólicas, incluindo aumento na glicólise, estresse oxidativo e detoxificação.

Análise proteômica de linhagens de *L. panamensis* sensíveis e resistentes ao Sb^{III} demonstrou uma alteração nos parasitos resistentes quanto aos níveis das proteínas envolvidas em resposta geral ao estresse, funções metabólicas e de transporte. As enzimas envolvidas no metabolismo de aminoácidos sulfurados, como S-adenosilmetionina sintetase e S-adenosilhomocisteína hidrolase, que são precursoras da síntese de tripanotiona, estavam superexpressas nas amostras resistentes ao Sb^{III}. Esse dado sugere um envolvimento destas enzimas na regulação em resposta à pressão de droga, reforçando a importância do metabolismo de tióis na resistência ao antimônio em *Leishmania* (Walker et al., 2012).

Dados de análise proteômica comparativa de linhagens de *L. braziliensis* e *L. infantum* sensíveis e resistentes *in vitro* ao Sb^{III} mostraram que a maioria das proteínas com abundância diferencial em ambas as espécies está envolvida na defesa antioxidante, resposta geral ao estresse, metabolismo de glicose e de aminoácidos, e organização do citoesqueleto. Cinco proteínas foram mais abundantes em ambas as linhagens de *Leishmania* resistentes ao Sb^{III}: triparedoxina peroxidase, alfa-tubulina, HSP60, HSP70 e HSP83. É interessante ressaltar que 40 proteínas foram observadas apenas nessa análise proteômica. Dessa maneira, os autores acreditam que algumas das proteínas identificadas nesse estudo estejam associadas com a resposta geral ao estresse e que outras podem estar associadas ao mecanismo de resistência ao antimônio trivalente em *Leishmania* (Matrangolo et al., 2013).

Apesar dos mecanismos de resistência aos antimoniais não serem totalmente elucidados, os trabalhos proteômicos mencionados anteriormente contribuem para uma maior compreensão de tais mecanismos tanto nas linhagens resistentes selecionadas *in vitro* quanto nos isolados clínicos. Sendo assim, os dados obtidos com esses estudos permitem a descoberta e validação de novos alvos para a quimioterapia das leishmanioses.

1.9 Modificações pós-traducionais

A identificação de proteínas que sofrem modificações pós-traducionais (MPTs) é uma das vantagens da análise proteômica, uma vez que estas modificações não são detectadas através de análise do genoma. MPTs como metilação, acetilação, glicosilação e fosforilação são capazes de regular a conformação proteica, função, estabilidade, ativação, degradação, localização e interações entre proteínas (Rosenzweig et al., 2008; White, 2008; Cuervo et al., 2010). Sendo assim, as MPTs são determinantes na complexidade dos organismos.

São conhecidos mais de 200 tipos de MPTs (Krishna & Wold, 1998), dos quais apenas alguns são reversíveis e importantes para a regulação de processos biológicos. Uma das MPTs mais estudadas é a fosforilação de proteínas (Raggiaschi et al., 2005). Em 1955, ela foi descrita pela primeira vez por Edwin Krebs e Edmond Fischer como sendo indispensável para a conversão de glicogênio fosforilase de uma forma inativa para uma forma ativa (Fischer & Krebs, 1955). Estima-se que cerca de um terço do proteoma de eucariotos seja fosforilado (Cohen, 2000). A fosforilação está envolvida em diversos processos celulares, como metabolismo, transcrição, tradução, progressão do ciclo celular, proliferação, rearranjo do citoesqueleto, apoptose, movimento e diferenciação celular. Além disso, a fosforilação mostra um papel importante na transferência de informação dentro da célula, regulando vias de transdução de sinais, comunicação intercelular durante o desenvolvimento, na ativação do sistema imune e no funcionamento do sistema nervoso (Raggiaschi et al., 2005). O tipo mais comum de fosforilação é a O-fosforilação, que pode ocorrer nos aminoácidos serina, treonina e tirosina, com uma proporção de aproximadamente 1.000/100/1, respectivamente (Hunter, 1998). Além deste tipo de fosforilação, resíduos de histidina, arginina e lisina podem sofrer N-fosforilação, enquanto ácido aspártico e ácido glutâmico podem ser acil-fosforilados e cisteína pode ser S-fosforilada (Sickamm & Meyer, 2001).

1.9.1 Fosfoproteoma

Fosfoproteoma corresponde ao conjunto das proteínas fosforiladas de um determinado organismo. Técnicas proteômicas podem ser utilizadas para o estudo do fosfoproteoma, permitindo a análise de todas as proteínas fosforiladas de uma

célula ao mesmo tempo ao invés de estudar uma única proteína. Todavia, a análise do fosfoproteoma apresenta dificuldades, como a complexidade da amostra, a faixa dinâmica de concentração das proteínas e mudanças que podem ocorrer ao longo do tempo (Nita-Lazar et al., 2008). A análise fosfoproteômica tem sido importante em diversas áreas da pesquisa científica, como na identificação de proteínas fosforiladas relacionadas com doenças, incluindo câncer (Blume-Jensen & Hunter, 2001), distúrbios metabólicos (Taniguchi et al., 2006), doenças autoimunes (Gatzka & Walsh, 2007) e infecções patogênicas (Sirard et al., 2007), na compreensão de redes de sinalização celular (Nita-Lazar et al., 2008; White, 2008) e efeito de drogas em diferentes vias (Tedford et al., 2009).

A análise do primeiro fosfoproteoma das formas promastigotas e amastigotas de *L. donovani* foi realizada por Morales et al. (2008), revelando fosfoproteínas envolvidas em resposta ao estresse e choque térmico, renovação de RNA/proteína, metabolismo, sinalização e proteínas hipotéticas. Os autores identificaram 73 fosfoproteínas, das quais 55 (75%) eram conservadas em *Leishmania* e 18 (25%) hipotéticas. Foi observado também que 35 (48%) destas proteínas não foram identificadas em estudos proteômicos prévios utilizando extratos de proteínas totais. Isso demonstra a importância do processo de purificação de fosfoproteínas para análise dessas proteínas de baixa abundância. As análises desses autores revelaram um subconjunto de fosfoproteínas que apresentaram fosforilação estágio-específico, incluindo proteínas ribossomais em promastigotas, e cinco proteínas que estavam fortemente ou exclusivamente fosforiladas no estágio amastigota, incluindo HSP100, MAP quinase de *Leishmania* homóloga à LmaMPK10, nucleosídeo difosfato quinase b, ciclofilina 40 e uma proteína hipotética (LmjF32.2260). Análise posterior realizada por Morales et al. (2010) aumentou o número de fosfoproteínas identificadas para 171 e revelou que 38% destas proteínas apresentaram diferenças significativas estágio-específico. Em amastigotas, foi observado que as fosfoproteínas estavam amplamente envolvidas na função chaperona, incluindo proteína induzida por estresse (STI1/HOP) e várias isoformas de HSP70 e HSP90, sugerindo que estas proteínas sejam reguladas pós-traducionalmente pela fosforilação.

Análise fosfoproteômica também tem possibilitado a identificação de sítios de fosforilação em amostras de parasitos. Hem et al. (2010) identificaram 445 fosfoproteínas associadas às funções biológicas de resposta ao estresse, renovação

de RNA/proteína e transdução de sinal em amastigotas axênicos de *L. donovani*. Análises de fosfopeptídeos enriquecidos com dióxido de titânio (TiO_2) identificaram 157 fosfopeptídeos únicos cobrindo 181 sítios de fosforilação em 126 proteínas distintas. Foi observado que 78% dos peptídeos estavam fosforilados em um resíduo, enquanto 19 e 3% dos peptídeos apresentaram fosforilações em dois e três resíduos, respectivamente. Os resultados mostraram que 86% dos sítios fosforilados ocorrem em resíduos de serina, enquanto 12 e 2% ocorrem em resíduos de treonina e tirosina, respectivamente. Esses dados revelam que a maioria dos sítios de fosforilação em *L. donovani* ocorre em resíduos de serina de forma similar a outros eucariotos. Investigação da conservação de sítios de fosforilação em tripanossomatídeos e eucariotos superiores através de análises de alinhamento de sequências múltiplas mostraram fosforesíduos em proteínas altamente conservadas que compartilham homologia de sequência a ortólogos do hospedeiro humano. Esses únicos sítios de fosforilação revelam importantes diferenças entre a biologia do hospedeiro e parasito e regulação pós-traducional de proteínas, que devem ser exploradas para a identificação de novos alvos quimioterápicos.

Análises comparativas dos fosfoproteomas de amastigotas e promastigotas de *L. donovani* demonstraram 1.614 sítios de fosforilação correspondentes a 627 proteínas (Tsigankov et al., 2013). Os autores observaram que 616 sítios (38%) foram identificados em amastigotas, 568 (35%) em promastigotas e 430 (27%) em ambos os estágios, indicando que a maioria desses sítios é estágio-específico. Além disso, a distribuição de aminoácidos fosforilados assemelha-se à de eucariotos superiores e *Leishmania*: 80% (serina), 19,6% (treonina) e 0,4% (tirosina). A fosforilação de serina no motivo específico “SF” (serina-fenilalanina) de tripanossomatídeos estava significativamente enriquecida em amastigotas. Das 627 fosfoproteínas identificadas nas análises comparativas, 322 apresentaram um único sítio de fosforilação, 155 possuíam dois sítios, enquanto que 57 e 23 proteínas tinham, respectivamente, três e quatro sítios distintos. As análises indicaram que *Leishmania* contém proteínas com múltiplos sítios de fosforilação que estão fosforilados em distintos estágios do ciclo de vida do parasito. Para mais da metade dos eventos de fosforilação, mudanças na abundância de fosfoproteínas não correlacionaram positivamente com alterações na abundância de proteínas, sugerindo regulação funcional.

Tsigankov et al. (2014) analisaram mudanças no perfil de fosforilação durante a diferenciação da forma promastigota à forma amastigota de *L. donovani*. O curso de tempo dessa diferenciação pode ser dividido em quatro fases morfologicamente distintas: I, percepção do sinal (0-5 h após exposição de promastigotas ao sinal de diferenciação, como 37ºC e pH 5,5); II, interrupção do movimento e agregação (5-10 h); III, formação de amastigota (10-24 h); e IV, maturação (24-120 h). Análises dos 163 fosfopeptídeos correspondentes a 106 proteínas revelaram diferentes perfis de cinética, com aumento na fosforilação predominante durante as fases I e III, enquanto que as fases II e IV foram caracterizadas por maior defosforilação. Várias proteínas estavam fosforiladas na fase I após exposição ao completo sinal de diferenciação. Várias outras proteínas quinases, incluindo subunidades regulatórias, e fosfatases também mostraram mudanças na fosforilação durante a diferenciação. Dessa maneira, as análises fosfoproteômicas podem contribuir para elucidar vias de sinalização em tripanossomatídeos, além de permitir a identificação de potenciais alvos de drogas para serem utilizados no desenvolvimento de novos agentes quimioterápicos.

1.9.1.1 Eletroforese em gel diferencial bidimensional (2D-DIGE)

Vários aprimoramentos dentro da metodologia de eletroforese bidimensional (2DE) foram realizados, dentre eles a eletroforese em gel diferencial bidimensional (2D-DIGE – *two dimensional difference gel electrophoresis*). Esta técnica foi descrita pela primeira vez por Unlü et al. (1997). Ela envolve a marcação de extratos proteicos com os diferentes marcadores fluorescentes: Cy2, Cy3 e Cy5 (CyDye DIGE fluor dyes, GE). Estes corantes, que são moléculas espectralmente resolvidas e equiparadas quanto à carga e massa, possuem um grupo reativo NHS-éster que se liga covalentemente aos resíduos de lisinas presentes nas proteínas. Então, as amostras marcadas são misturadas, e a primeira e segunda dimensões são realizadas em um único gel 2DE. Os extratos proteicos marcados com os diferentes corantes podem ser visualizados individualmente através da excitação específica de cada um dos fluoróforos com diferentes comprimentos de ondas, que gera imagens digitais de cada amostra separadamente (Marouga et al., 2005).

Nesse tipo de experimento recomenda-se a utilização de um padrão interno, que deve estar presente em todos os géis de um experimento. Este padrão interno é

constituído por quantidades iguais de proteínas de cada amostra biológica do experimento, as quais são marcadas com um mesmo corante, geralmente o Cy2. Sendo assim, as proteínas de cada amostra estarão representadas no padrão interno, permitindo que elas sejam normalizadas com o padrão interno presente no mesmo gel. Com isso, é possível realizar uma quantificação mais robusta devido ao aumento da confidência do *matching* entre os géis, bem como separar variações induzidas biologicamente daquelas produzidas pela variação experimental (Marouga et al., 2005).

A técnica 2D-DIGE oferece maior sensibilidade na detecção de proteínas, sendo necessários apenas poucos microgramas de amostra em comparação à técnica 2DE convencional, apresentando assim grande importância para estudos que dispõem de pequenas quantidades de amostras. Nessa técnica, a corrida das amostras em um mesmo gel proporciona uma redução na variação do padrão dos *spots*, além de reduzir o número de géis necessários para validar estatisticamente o experimento (Marouga et al., 2005). Entretanto, essa técnica apresenta algumas desvantagens como: os corantes utilizados nas análises tornam a metodologia mais onerosa, a resolução das proteínas ainda é difícil em faixas extremas de pH e massa molecular, além da possibilidade de um mesmo *spot* apresentar mais de uma proteína e a limitação de se estudar proteínas de membrana cujos protocolos de extração são realizados com detergentes que interferem na isoeletrofocalização.

Atualmente, muitos grupos de pesquisa têm utilizado esse tipo de análise proteômica para identificar marcadores de proteína específicos em câncer (Zhou et al., 2002), fosfoproteínas estágio-específico em *L. donovani* (Morales et al., 2010), antígenos candidatos para o desenvolvimento de testes diagnósticos e vacina para leishmaniose visceral canina (Costa et al., 2011), biomarcadores de prognóstico para leishmaniose visceral humana (Rukmangadachar et al., 2011), proteínas diferencialmente expressas em linhagens de *Toxoplasma gondii* sensíveis e resistentes à sulfadiazina (Doliwa et al., 2013), fatores de virulência em amostras de *L. infantum* (da Fonseca Pires et al., 2014), análise proteômica comparativa de isolados de *L. infantum* sensíveis e resistentes à miltefosina (Carnielli et al., 2014), dentre outros. Dessa forma, considerando as vantagens que a metodologia 2D-DIGE apresenta, como melhor reprodutibilidade, sensibilidade e maior robustez dos resultados quando comparada às outras metodologias proteômicas, esta técnica tem se tornado uma ferramenta útil para avaliações moleculares que ocorrem em

diversos sistemas biológicos, bem como para análise de doenças e potenciais terapias (Kim et al., 2009).

1.10 Alvos selecionados para o estudo de genômica funcional

Os resultados de nossa análise fosfoproteômica demonstraram que as proteínas nucleosídeo difosfato quinase b (NDKb) e fator de elongação 2 (EF2) estavam mais e menos abundantes, respectivamente, na linhagem de *L. braziliensis* resistente ao Sb^{III} em comparação à sua respectiva linhagem sensível (Moreira et al., 2015). Dessa forma, estas proteínas foram selecionadas para a realização da segunda etapa do projeto: a genômica funcional.

1.10.1 Nucleosídeo difosfato quinase b (NDKb)

A enzima nucleosídeo difosfato quinase b (NDKb), membro da família NDK, catalisa a transferência de grupo fosfato de um nucleosídeo trifosfato (NTP) para um nucleosídeo difosfato (NDP) através de um mecanismo “ping-pong”, no qual envolve fosfohistidina como intermediário (Parks et al., 1973; Lascu & Gonin, 2000). A reação completa pode ser representada da seguinte maneira (Paul et al., 2014):

$$\text{NDK} + \text{N}_1\text{TP} \leftrightarrow \text{NDK-P} + \text{N}_1\text{DP} \leftrightarrow \text{NDK-P} + \text{N}_2\text{DP} \leftrightarrow \text{NDK} + \text{N}_2\text{TP}$$

Essa enzima é essencial para a manutenção dos níveis de NTP intracelular e para o fornecimento de NTPs para a síntese de ácidos nucleicos, citidina trifosfato (CTP) para síntese de lipídeos, uridina-5’-trifosfato (UTP) para síntese de polissacarídeos e guanosina-5’-trifosfato (GTP) para elongação de proteínas, transdução de sinal e polimerização de microtúbulos (Tonoli et al., 2009). Outras funções têm sido atribuídas à NDK em diferentes organismos, incluindo patogênese bacteriana (Chakrabarty, 1998), regulação da expressão gênica em mamíferos (Postel, 2003) e participação nas vias de recuperação de purinas em tripanossomatídeos (Landfear et al., 2004). NDKb foi identificado como um componente abundante na análise subproteômica da fração microssomal de promastigotas de *L. major* (de Oliveria et al., 2006). Kolli et al. (2008) demonstraram

que NDKb é secretada por *Leishmania* spp. durante a infecção, impedindo a lise de macrófagos mediada por ATP e preservando a integridade da célula hospedeira para benefício do parasito. Dessa maneira, a enzima NDKb pode ser considerada um potencial alvo para o desenvolvimento de novas estratégias para o tratamento das leishmanioses.

A enzima NDKa de humano (NM23-H1) age como um supressor de metástase tumoral em células de melanoma (Lacombe et al., 2000). Por outro lado, a enzima NDKb de humano (NM23-H2) foi descrita como um ligante da região promotora do oncogene *c-myc*, resultando na ativação da transcrição (Harstsough & Steeg, 2000). Além disso, NDK de *Escherichia coli* tem uma função no sistema de reparo de DNA (Postel & Abramczyk, 2003) e uma atividade exonuclease 3'-5' em NDK1 de humano também tem sido demonstrada (Yoon et al., 2005). Estudo proteômico relatou uma abundância maior da proteína NDK em população de *Trypanosoma cruzi* resistente ao benzonidazol (Andrade et al., 2008). Dessa forma, os estudos mencionados demonstram que NDKb é uma enzima ubíqua que está envolvida em diversos processos celulares em diferentes organismos.

1.10.2 Fator de elongação 2 (EF2)

A síntese de proteínas é constituída por três etapas gerais: iniciação, elongação e terminação. Essa síntese está sujeita à regulação complexa que permite as células controlarem este processo intenso de gasto energético em resposta a diversos estímulos fisiológicos (Hizli et al., 2013). A tradução é regulada nos níveis de iniciação e elongação. Por exemplo, a tradução é reprimida na fase G_2/M do ciclo celular (Celis et al., 1990; Pyronnet & Sonenberg, 2001), na qual a inibição de fatores de iniciação eucariótico reprimem a tradução mitótica (Fan & Penman, 1970; Bonneau & Sonenberg, 1987; Pyronnet & Sonenberg, 2001). O controle traducional ocorre também ao nível de elongação, o que envolve a inibição do fator de elongação eucariótico 2 (EF2) (Browne & Proud, 2002; Jørgensen et al., 2006; Sivan et al., 2007).

EF2 é uma translocase dependente de GTP, que é responsável pelo movimento do nascente peptidil-tRNA do sítio A (aminoacil) para o sítio P (peptidil) do ribossomo (Hizli et al., 2013). Um mecanismo que inibe a elongação é a fosforilação de EF2 na treonina 56 (T56) pela quinase EF2K (Price et al., 1991), que,

consequentemente, inibe a síntese de proteínas. Dados da literatura têm demonstrado uma alta expressão de EF2 em uma variedade de tumores malignos, como câncer de ovário (Alaiya et al., 1997), carcinoma hepatocelular (Li et al., 2008), câncer gastrointestinal humano (Nakamura et al., 2009) e adenocarcinoma de pulmão (Chen et al., 2011). Além disso, a superexpressão de EF2 está também correlacionada com a progressão da célula cancerosa e recorrência tumoral precoce (Nakamura et al., 2009; Oji et al., 2014). Outros estudos relataram o aumento na abundância de EF2 em isolados de *L. donovani*, e em linhagens de *L. panamensis* e *L. infantum* resistentes aos antimoniais (Biyani et al., 2011; Walker et al., 2012; Matrangolo et al., 2013).

2 JUSTIFICATIVA

As leishmanioses são um grande problema de saúde pública mundial. Todavia, poucos avanços foram obtidos em seu controle. Sendo assim, a quimioterapia continua sendo uma das principais medidas utilizadas para o controle de todas as formas da doença. Entretanto, os fármacos utilizados no tratamento são tóxicos e ainda existe a possibilidade de surgimento de parasitos resistentes às drogas. Nesse sentido, a busca pela compreensão dos mecanismos bioquímicos e moleculares que contribuem para o fenótipo de resistência em espécies de *Leishmania* tem sido objeto de diversas pesquisas científicas mundiais nos últimos anos. Contudo, a grande maioria dos estudos realizados relata a resistência aos antimoniais em espécies de *Leishmania* do Velho Mundo, tornando-se necessário o entendimento desse fenômeno nas espécies predominantes no Novo Mundo.

Análises proteômicas têm sido muito utilizadas para determinar o perfil proteico de diversos parasitos, principalmente em *Leishmania*. Tais estudos têm permitido avaliar as alterações na expressão de proteínas em resposta à exposição de drogas, bem como contribuído para o aumento da compreensão de mecanismos associados ao fenótipo de resistência aos antimoniais. Nesse contexto, métodos proteômicos são de grande relevância pelo fato de possibilitarem a identificação de novos alvos moleculares para utilização em processos de validação e desenvolvimento de novas drogas para serem utilizadas no tratamento da doença.

Modificações pós-traducionais apresentam um papel importante na estabilidade de proteínas, além de contribuir para o controle da expressão gênica. Tais modificações devem regular a função de uma proteína pela alteração de sua localização, interação molecular ou atividade. A fosforilação de proteínas é uma dessas modificações mais estudadas devido à sua importância em diversos eventos celulares. Entretanto, a literatura não havia relatado estudo sobre o perfil de fosfoproteínas em amostras de *L. braziliensis* resistentes ao antimônio trivalente. Dessa forma, nesse trabalho foi realizada uma análise fosfoproteômica comparativa, utilizando linhagens sensível e resistente ao Sb^{III} desta espécie de *Leishmania*, as quais foram previamente selecionadas *in vitro* pelo nosso grupo de pesquisa (Liarte & Murta, 2010). Esse estudo fosfoproteômico permitiu a identificação de proteínas diferencialmente abundantes associadas ao estresse causado pelo tratamento de droga e ao fenótipo de resistência ao antimônio trivalente.

A partir dos resultados obtidos na análise fosfoproteômica, as proteínas nucleosídeo difosfato quinase b (NDKb) e fator de elongação 2 (EF2) foram selecionadas para estudos de genômica funcional. Estas fosfoproteínas apresentaram maior e menor abundância, respectivamente, na linhagem de *L. braziliensis* resistente ao Sb^{III} comparada ao seu respectivo par sensível (Moreira et al., 2015). NDKb é uma enzima multifuncional com papel essencial na síntese de nucleosídeos trifosfatos e no metabolismo de purinas em *Leishmania*. Já a enzima EF2 é crucial na elongação e síntese de proteínas do parasito. Dessa maneira, foi realizada a superexpressão dessas enzimas em *L. braziliensis* e *L. infantum* para investigar o envolvimento dessas proteínas no fenótipo de resistência ao antimônio trivalente nestes parasitos. Além disso, inibidores foram utilizados para avaliar a susceptibilidade ao Sb^{III} em parasitos superexpressores das enzimas NDKb e EF2. Portanto, nossos estudos poderão subsidiar a seleção de novos alvos para o desenvolvimento de estratégias que possam ser utilizadas no tratamento das leishmanioses.

3 OBJETIVOS

3.1 Objetivo geral

Realizar análise fosfoproteômica e genômica funcional de linhagens de *Leishmania* spp. sensíveis e resistentes ao antimônio trivalente, visando entender os mecanismos moleculares que contribuem para o fenótipo de resistência dos parasitos a esse fármaco.

3.2 Objetivos específicos

A) Análise fosfoproteômica:

- ✓ Determinar o perfil das proteínas fosforiladas das linhagens de *L. braziliensis* sensível e resistente ao antimônio trivalente;
- ✓ Identificar as proteínas diferencialmente fosforiladas por espectrometria de massas;
- ✓ Realizar análises *in silico* para validar a conservação de sítios de fosforilação em algumas proteínas identificadas;
- ✓ Analisar os níveis de expressão de fosfoproteínas nas linhagens de *L. braziliensis* sensível e resistente ao Sb^{III}.

B) Genômica funcional:

- ✓ Inserir os genes *NDKb* e *EF2* no plasmídeo de expressão de *Leishmania* (pIR1BSD);
- ✓ Transfectar as linhagens de *L. braziliensis* e *L. infantum* com os plasmídeos contendo os genes *NDKb* e *EF2*;
- ✓ Confirmar a presença do marcador de seleção (blasticidina) e aumento de expressão das proteínas NDKb e EF2;
- ✓ Determinar a susceptibilidade dos parasitos transfectados ao antimônio trivalente;

- ✓ Analisar o efeito do inibidor lamivudina e sua associação com o Sb^{III} no crescimento das linhagens de *L. braziliensis* sensível e superexpressora de NDKb;
- ✓ Determinar a susceptibilidade das linhagens de *L. braziliensis* sensível e superexpressora de NDKb ao peróxido de hidrogênio;
- ✓ Avaliar o efeito do inibidor de EF2K e sua associação com o Sb^{III} no crescimento das linhagens de *L. braziliensis* sensível e superexpressora de EF2;
- ✓ Analisar os níveis de expressão das proteínas NDKb e EF2 nas linhagens de *L. braziliensis* e *L. infantum* sensíveis e resistentes ao antimônio trivalente.

4 ARTIGOS

4.1 Artigo 1: Análise fosfoproteômica

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RESEARCH ARTICLE

Phosphoproteomic analysis of wild-type and antimony-resistant *Leishmania braziliensis* lines by 2D-DIGE technology

Douglas de Souza Moreira[1], Pascale Pescher[2], Christine Laurent[3], Pascal Lenormand[3], Gerald F. Späth[2] and Silvane M. F. Murta[1]

[1] Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou CPqRR/Fiocruz, Belo Horizonte, MG, Brazil
[2] Institut Pasteur, CNRS URA2581, Unité de Parasitologie Moléculaire et Signalisation, Paris, France
[3] Department of Structural Biology and Chemistry, Pasteur-Genopole Ile-de-France, Plate-forme de Protéomique, Institut Pasteur, Paris, France

Protein phosphorylation is one of the most studied post-translational modifications that is involved in different cellular events in *Leishmania*. In this study, we performed a comparative phosphoproteomics analysis of potassium antimonyl tartrate (SbIII)-resistant and -susceptible lines of *Leishmania braziliensis* using a 2D-DIGE approach followed by MS. In order to investigate the differential phosphoprotein abundance associated with the drug-induced stress response and SbIII-resistance mechanisms, we compared nontreated and SbIII-treated samples of each line. Pair wise comparisons revealed a total of 116 spots that showed a statistically significant difference in phosphoprotein abundance, including 11 and 34 spots specifically correlated with drug treatment and resistance, respectively. We identified 48 different proteins distributed into seven biological process categories. The category "protein folding/chaperones and stress response" is mainly implicated in response to SbIII treatment, while the categories "antioxidant/detoxification," "metabolic process," "RNA/DNA processing," and "protein biosynthesis" are modulated in the case of antimony resistance. Multiple sequence alignments were performed to validate the conservation of phosphorylated residues in nine proteins identified here. Western blot assays were carried out to validate the quantitative phosphoproteome analysis. The results revealed differential expression level of three phosphoproteins in the lines analyzed. This novel study allowed us to profile the *L. braziliensis* phosphoproteome, identifying several potential candidates for biochemical or signaling networks associated with antimony resistance phenotype in this parasite.

Keywords:
Antimony resistance / 2D-DIGE / *L. braziliensis* / Microbiology / Phosphoproteomic analysis / SbIII

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1 Introduction

Protozoan parasites belonging to *Leishmania* genus are the causative agents of leishmaniasis that produces a wide spectrum of clinical disease in humans ranging from self-healing cutaneous (CL) and mucocutaneous (MCL) lesions to fatal visceral (VL) infection, if not treated [1]. The disease is a public health concern, endemic in 98 countries with more than

Correspondence: Dr. Silvane M. F. Murta, Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou CPqRR/Fiocruz, Av. Augusto de Lima 1715, CEP 30190-002, Belo Horizonte, MG, Brazil
Fax: +55-31-32953566
E-mail: silvane@cpqrr.fiocruz.br

Abbreviations: **Lb**, *L. (V.) braziliensis*; **SbIII**, potassium antimonyl tartrate; **SbR**, SbIII-resistant; **WTS**, wild-type susceptible

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350 million people at risk, reaching up to 1.2 million new cases annually [2]. In the New World, *Leishmania (Viannia) braziliensis* (*Lb*) causes the CL and MCL forms of the disease [3,4]. Cases of CL occur mainly in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia, the Syrian Arab Republic and Tunisia, whereas MCL occurs mainly in Brazil, Peru, and the Plurinational State of Bolivia [2].

Chemotherapy remains the only effective measure against all forms of leishmaniasis. The administration of the pentavalent antimony (SbV) -based drugs, such as sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) represents the first line of treatment for more than 60 years [5]. However, the mechanisms of action of antimonials are not completely understood. It is generally accepted that SbV is a prodrug that is reduced into the trivalent form (SbIII), active against the amastigote, and promastigote forms of *Leishmania* [6]. Nevertheless, the site (macrophage or parasite) and the mechanism of this reduction (enzymatic or nonenzymatic) remain unclear. Studies suggest that SbV inhibits macromolecular biosynthesis in amastigotes [7], possibly via perturbation of energy metabolism due to inhibition of glycolysis and fatty acid oxidation [8]. Moreover, other studies have been reported that antimony can kill *Leishmania* by disturbances in the thiol redox potential of the parasite [9], by production of leishmanicidal molecules like nitric oxide and reactive oxygen species [10], DNA fragmentation and programmed cell death [11,12].

An increase in clinical resistance to SbV has been reported [13,14], representing an important public health problem in several countries. In India, as many as 65% of previously untreated patients failed to respond after therapy with antimony drugs due to the development of drug resistance [15,16]. The phenomenon of antimony resistance in *Leishmania* species is complex, multifactorial, and involves several pathways. Thus, different mechanisms have been suggested for drug resistance such as gene amplification, reduced accumulation of the active drug due to either decreased influx or increased efflux, detoxification of toxic compounds, and sequestration of actives molecules in intracellular vesicles of the parasite [17].

Since the regulation of gene expression in trypanosomatids occurs largely at posttranscriptional levels, proteomic approaches have been employed to detect alterations of protein expression in *Leishmania* species [18–20]. Indeed, previous studies used proteome profiling of Old- and New-World *Leishmania* species to investigate virulence factors, potential drug targets, and the mechanisms of drug resistance in these parasites [21–24].

During differentiation, *Leishmania* proteins undergo posttranslational modifications (PTMs), which can include methylation, glycosylation, fucosylation, acetylation, and phosphorylation [25]. However, protein phosphorylation is one of the most studied modifications given its relevance in regulating important cellular events such as gene transcription, cell morphology, or cell cycle [26]. Thus, phosphoproteomic analyses have been performed to identify phosphoproteins implicated in several biological process of *L. donovani* pro- and amastigote stages [27], to reveal heat-shock protein complexes specific to the *L. donovani* infectious stage [28], define parasite-specific phosphorylation sites [29], identify a stage-specific phosphorylation motif in *L. donovani* parasites [30], and follow the dynamics of phosphorylation trends during *L. donovani* axenic differentiation [31]. Our study is the first report in the literature of comparative phosphoproteomic analysis associated with antimony resistance in *L. braziliensis*. Here, we used a quantitative 2D-DIGE approach followed by MS/MS to correlate phosphoprotein abundance in SbIII-resistant (SbR) and -susceptible lines to either drug treatment (SbIII 0.025 or 2 mg/mL) or antimony resistance. Western blot assays were carried out to validate the quantitative phosphoproteome analysis. Thus, we investigated the expression level of three phosphoproteins, such as cytosolic tryparedoxin peroxidase, kinetoplastid membrane protein-11, and alpha-tubulin in these *L. braziliensis* lines. In addition, multiple sequence alignments were performed to validate the conservation of phosphorylated residues in nine proteins identified in this study.

2 Materials and methods

2.1 *Leishmania* samples and parasite growth

In this study, we used promastigote forms (mid-log) from the lines of *L. braziliensis* (MHOM/BR/75/M2904) that are susceptible or resistant to potassium antimonyl tartrate (SbIII). The resistant line (LbSbR) was previously selected in vitro by step-wise increase of SbIII drug pressure and the resistance index is 20-fold higher than of its susceptible counterpart [32]. The authors showed that after 37 passages in culture medium in the absence of SbIII, the resistance index decreased to half (tenfold) for these resistant parasites. No further decrease in resistance was observed in parasites grown for ten more passages, demonstrating the stability of the SbIII resistance phenotype. Moreover, a decrease in infectivity and growth profiles was observed in the LbSbR parasites. Lesions in mice infected with these parasites appeared after 37 days, progressed more slowly and peaked at a lower thickness than lesions produced by LbWTS parasites [32]. The SbIII concentrations used in this study were obtained after detailed analysis of parasite viability. Different SbIII concentrations were used and the parasites were submitted to FACS analysis, using propidium iodide and annexin V (ImmunoChemistry Technologies). The antimony-susceptible line (LbWTS) was cultivated in the absence of the drug (LbWTS 0 sample) or during 24 h at a SbIII concentration of 0.025 mg/mL (LbWTS 0.025 sample) that corresponds to half of the SbIII IC_{50} for this LbWTS line, which does not induce apoptosis (Supporting Information Fig. 1A). For comparative analysis, the LbSbR line was also cultivated during 24 h at the concentrations of 0.025 mg/mL (LbSbR 0.025 sample—same SbIII concentration and half of the SbIII IC_{50} of LbWTS line) and

2 mg/mL that corresponds to the SbIII IC_{50} for this LbSbR line and it is the concentration that the parasite is routinely maintained (LbSbR 2 sample) (Supporting Information Fig. 1B). It is important to highlight that we used the same SbIII concentration (0.025 mg/mL) to compare the wild-type susceptible (WTS) and SbR lines, and to evaluate if the stress caused by the drug was also present in the SbR line maintained with high concentration of SbIII (2 mg/mL). Parasites were grown at 26°C in M199 medium supplemented with 40 mM HEPES pH 7.4, 1 μg/mL biotin, 5 μg/mL hemin, 2 μg/mL biopterin, 2 mM L-glutamine, 500 U penicillin, 50 μg/mL streptomycin and 10% v/v heat-inactivated fetal calf serum [32]. Cells were washed in RPMI medium, pelleted by centrifugation and frozen at –70°C.

2.2 Preparation of total protein and phosphoprotein extracts of *L. braziliensis* samples

Total protein and phosphoprotein extracts were prepared as previously described [27, 33]. To obtain total protein extracts, promastigote pellets of *L. braziliensis* were resuspended in 0.7 mL lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, protease inhibitor cocktail (Roche), pH 8.5), incubated for 10 min at 4°C and sonicated 5 min (sequence of 10 s pulse and 20 s pause). Cells were centrifuged at 12 000 × *g* for 10 min at 4°C and the protein concentration was determined (*RC DC*™ Protein Assay BIORAD kit). For phosphoprotein purification, promastigote pellets were resuspended in 0.7 mL lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% TritonX-100, 0.25% CHAPS, 1% benzonase, protease inhibitor cocktail (Roche), and phosphatase inhibitor (PhosSTOP Roche)). Samples were incubated for 10 min at 4°C, sonicated 5 min (sequence of 10 s pulse and 20 s pause) and centrifuged. Protein concentration was adjusted to 0.1 mg/mL and the extracts were applied onto equilibrated affinity columns of the Phosphoprotein Purification kit (Qiagen) according to the manufacturer's instructions. Unbound proteins were removed by washing, and bound phosphorylated proteins were eluted in 1 mL elution buffer. Samples were concentrated using AMICON ultra centrifugation filters (0.5 mL, 10 kDa cut off), protein concentration was determined (*RC DC*™ Protein Assay BIORAD kit) and phosphoprotein extracts were kept at –70°C until use.

2.3 2D-DIGE analysis

2.3.1 Sample preparation and DIGE labeling

The analysis were performed following the manufacturer's recommendations (GE Healthcare) and as previously described [28, 33]. Briefly, phosphoprotein extracts from antimony-susceptible and -resistant *L. braziliensis* samples of four independent experimental replicates were precipitated using the 2D clean-up kit (GE Healthcare) and pellets were resuspended in DIGE sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5) to a final protein concentration of 5 mg/mL. Forty micrograms of phosphoproteins from each replicate were differentially labeled with the spectrally resolvable dyes Cy3 or Cy5 and mixed according to our experimental design. For normalization purposes an internal standard corresponding to Cy2 labeled protein representing a mixture of all extracts was added.

2.3.2 Two-dimensional gel electrophoresis

According to our experimental design (Fig. 1A and B), 120 μg of proteins from mixed samples were loaded on 24 cm DryStrip pH 4–7 Immobiline strips and separated in the first dimension using a isoelectric focusing system (BIORAD) at 60 kVHrs. After equilibration, strips were transferred to HPE NF flatbed gel (12.5%) (SERVA) and separated using the "High Performance Electrophoresis (HPE) FlatTop Tower" system (SERVA Electrophoresis) according to manufacturer's guidelines.

2.3.3 Image acquisition and protein identification by MS

Gels were scanned on a Typhoon™ Variable Mode Imager 9400 (GE Healthcare) and quantitative analysis of the images were performed using the Progenesis SameSpots software V4.1 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Validation of significant spots was further performed using the included Progenesis Stats DIGE module including Principal Component Analysis, computed *p* values using one way ANOVA analysis, Power and q value (Fig. 1C). Spots showing a minimum of 1.5-fold change in fluorescence intensity with a *p*-value less than 0.05 were considered significantly modulated. Selected spots were excised from the 2D-DIGE gels using the Screen Picker (Proteomics Consult), in order to maximize the amount of protein available for MS analysis, each spot was excised four times using the four available DIGE gels. Plugs were individually washed, reduced, alkylated, and digested with trypsin [34]. The resulting peptides were extracted with the Progest Investigator (Genomic Solutions, Ann Arbor, MI, USA). After desalting step (C18-μZipTip, Millipore), peptides were eluted and the four eluates corresponding to one same spot were pooled together onto the stainless steel MALDI target plate (Applied Biosystems/MDS SCIEX, Framingham, MA, USA) with 0.5 μL of CHCA matrix (2.5 mg/mL in 70% ACN/30% H_2O/0.1% TFA). The spots subjected to MS/MS were determined using a composite database of the *Leishmania brazilensis*, *L. mexicana*, *L. donovani*, *L. amazonensis*, and *L. major* (NCBI - http://www.ncbi.nlm.nih.gov and TriTrypDB- http://tritrypdb.org/tritrypdb/).

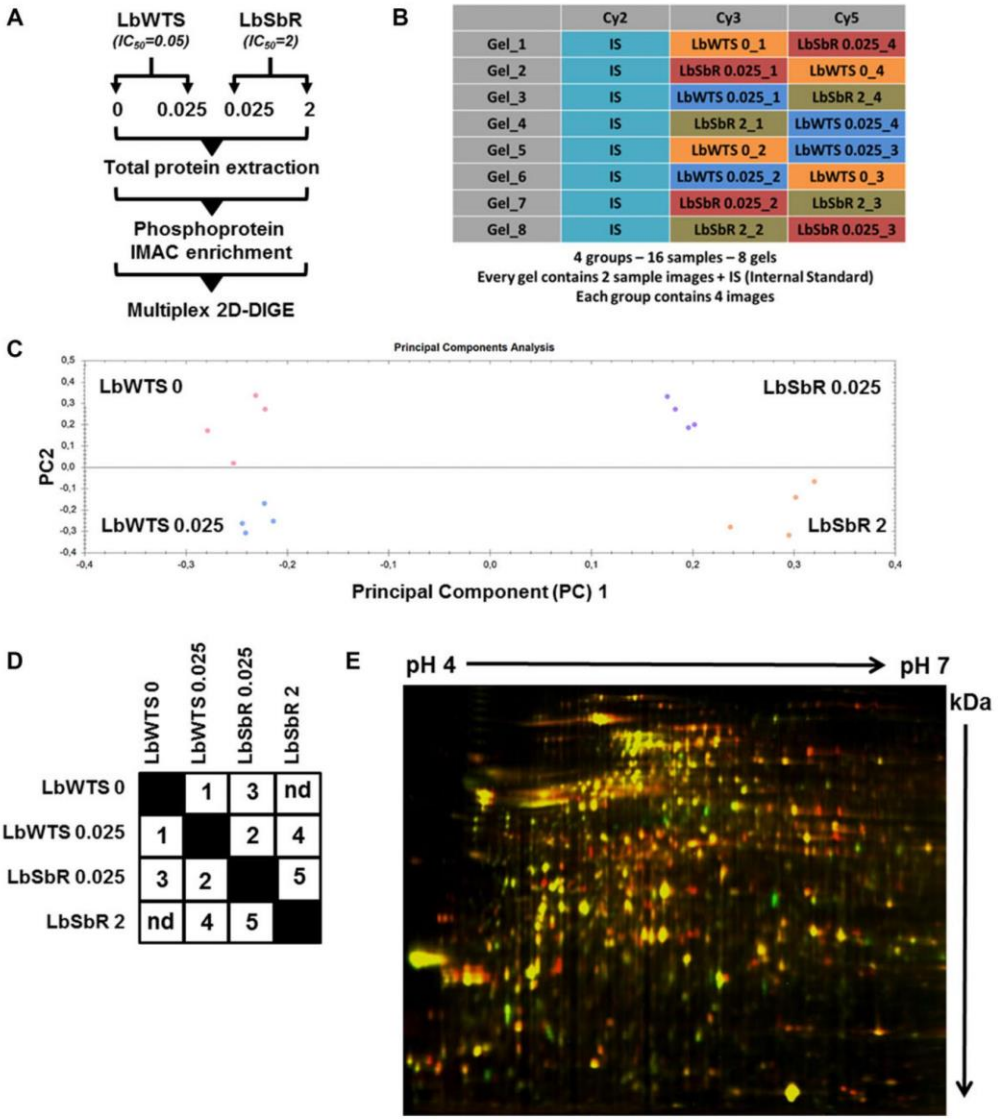

Figure 1. Experimental conditions for 2D-DIGE analysis of *L. braziliensis* samples. (A) Schematic. representation of the experiment. (B) Design of the analysis with randomized swap-dye—40 μg of each replicate were labeled with 400 pmoles of either Cy3 or Cy5 dyes and mixed according to our experimental design including the internal standard (IS). Each gel contained 120 μg in total. First dimensions were run for 60 kVHrs in 24 cm IPG strips, pH gradient 4–7, followed by second dimensions using the horizontal system HPE FlatTop Tower. (C) Principal components (PC) analysis of the 2D-DIGE experiment. Statistical analysis showing the "good" clustering of the biological replicates for each sample. (D) Experimental groups defined by pair-wise analyses. Group 1 (LbWTS 0 x LbWTS 0.025) and group 5 (LbSbR 0.025 x LbSbR 2) inform on pathways that are activated during drug treatment and thus reflects the parasite signaling response to stress by drug treatment; groups 2 (LbWTS 0.025 x LbSbR 0.025), 3 (LbWTS 0 x LbSbR 0.025), and 4 (LbWTS 0.025 x LbSbR 2) reveal differences between SbIII-susceptible and -resistant lines and thus may inform on resistance mechanisms. (E) Overlay image of the LbWTS 0.025 and LbSbR 2 samples by 2D-DIGE technology. Phosphoprotein extracts of the LbWTS 0.025 and LbSbR 2 samples were differentially labeled with fluorescent CyeDyes and subsequently separated on a 2D-DIGE gel using 24 cm pH 4–7 strips in the first dimension and 12.5% SDS-PAGE gels in the second dimension, and revealed with a Typhoon fluorescent imager. The gel showed above is representative of four independent experimental biological replicates. LbWTS 0, antimony-susceptible sample of *L. braziliensis* without treatment with SbIII; LbWTS 0.025, antimony-susceptible sample of *L. braziliensis* treated with SbIII 0.025 mg/mL; LbSbR 0.025, antimony-resistant sample of *L. braziliensis* treated with SbIII 0.025 mg/mL; LbSbR 2, antimony-resistant sample of *L. braziliensis* treated with SbIII 2 mg/mL.

2.4 Western blot

Total protein and phosphoprotein-enriched extracts (1 μg) of the different *L. braziliensis* samples treated and nontreated with SbIII were separated by electrophoresis on a 12% SDS polyacrylamide gel and electrotransferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The blots were blocked by incubation with 5% instant nonfat dry milk in PBS supplemented with 0.05% Tween 20 (PBS-T) for 1 h. Blots were washed twice in PBS-T and incubated for 16 h at 4°C with rabbit polyclonal antibodies specific for each protein of interest: *T. cruzi* anti-cytosolic tryparedoxin peroxidase (1:500) [35], *L. braziliensis* anti-KMP-11 (1:1000) (kindly provided by MSc. Nayara Tessarolo), and mouse monoclonal anti-alpha-tubulin antibody (1:500) (Sigma, St. Louis, USA). The blots were washed and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (1:3000) (GE Healthcare) or anti-mouse poly-HRP (1:1000) (Pierce) at room temperature. The membranes were washed, incubated with ECL Plus chemiluminescent substrate (GE Healthcare) and exposed to an X-ray film (Amersham, Buckinghamshire, UK) or revealed by ImageQuant LAS 4000 (GE Healthcare). To confirm equivalent loading, SDS-PAGE containing the same samples were stained with silver and Pro-Q Diamond phosphoprotein gel stain (Invitrogen). One region of the silver stained gel was selected to normalize the differences found in the results.

3 Results

3.1 Design and validation of the experimental set up for quantitative phosphoproteomics analysis

The aim of this study was to decipher potential signaling events linked to drug resistance mechanisms by the comparative analysis of the phosphoproteome of *L. braziliensis* lines susceptible and resistant to SbIII in response to the drug signal.

A preliminary experiment was designed to determine the nonlethal doses of antimony that will allow us to reveal signaling events in response to drug treatment and underlying the resistance phenotype rather than linked to cell death. Parasite viability was monitored by FACS analysis using propidium iodide across two SbIII concentrations for 24 and 48 h (Supporting Information Fig. 1A and B) and the following conditions were identified for subsequent quantitative phosphoproteomics investigation based on the absence of cell death and the presence of changes in the phosphoprotein profile (Supporting Information Fig. 1C): susceptible parasites were incubated for 24 h without (LbWTS 0) or with SbIII 0.025 mg/mL (LbWTS 0.025), and resistant parasites were treated with SbIII 0.025 mg/mL (LbSbR 0.025) and 2 mg/mL (LbSbR 2) before phosphoprotein enrichment (Fig. 1A).

For quantitative 2D-DIGE analysis, phosphoprotein extracts from four independent experimental biological replicates were differentially labeled with fluorescent CyeDyes, mixed according to our experimental design (Fig. 1B) and separated by isoelectric focusing at a pH range of 4–7 in the first and 12.5% HPE NF flatbed gel in the second dimension. The images were obtained by scanning on a Typhoon fluorescent imager, and analyzed with the Progenesis SameSpots software V4.1 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Before quantification, the experiment was controlled for quality with the QC module of the Progenesis SameSpots software. The principal component analysis applied on the gels is indicative of the reproducibility between replicates and the clear clusterization of the samples in four groups corresponding to LbWTS 0, LbWTS 0.025, LbSbR 0.025, and LbSbR 2 (Fig. 1C). Five groups of pairwise comparison were chosen that can inform on either on parasite signaling responses linked to drug treatment in general, namely groups 1 (LbWTS 0 × LbWTS 0.025) and 5 (LbSbR 0.025 × LbSbR 2), or signaling events associated to resistance mechanisms that are revealed in groups 2 (LbWTS 0.025 × LbSbR 0.025), 3 (LbWTS 0 × LbSbR 0.025), and 4 (LbWTS 0.025 × LbSbR 2) (Fig. 1D).

3.2 Pairwise comparison reveals phosphoproteins linked to antimony treatment and resistance

All spots with fold change above 1.5 and p-values < 0.05 were considered significantly modulated and submitted to MS. The pairwise comparisons of the gels (Fig. 1E and Supporting Information Fig. 2 to 5) reveal a total number of 116 spots that were found differentially abundant across the five groups (Fig. 2A). Of this total number, 34 spots showed fold changes above 2, and 11 and 34 spots were specific to groups of comparisons correlated to SbIII treatment and resistance, respectively (Tables 1–6). A total of 48 different proteins were identified by MS analysis, with some retrieved from several spots revealing isoforms or PTMs. Our results from comparative analyses demonstrated that the groups 1 and 5 may be related to drug response (Fig. 2B), and the groups 2, 3, and 4 associated to drug resistance (Fig. 2C). Besides, these graphs show the reproducibility between the four independent replicates under two conditions for each group analyzed. The various proteins that fall into these groups will be more explicitly described in the Sections 3.3 to 3.5. The resulting proteins were further classified in seven categories according to GO (http://geneontology.org/) and KEGG orthology (http://www.genome.jp/kegg/kegg2.html) databases of biological processes (Tables 2–6 and Supporting Information Table 1). Interestingly, we observed that the biological process category "protein folding/chaperones and stress response" is mainly implicated in response to SbIII drug as demonstrated for the groups 1 and 5 (Fig. 3A). Whilst "antioxidant/detoxification," "metabolic process," "RNA/DNA processing," and "protein biosynthesis" are modulated in the case of drug resistance for the groups 2, 3, and 4 (Fig. 3B).

Supporting Information Table 1 disponível na versão *online

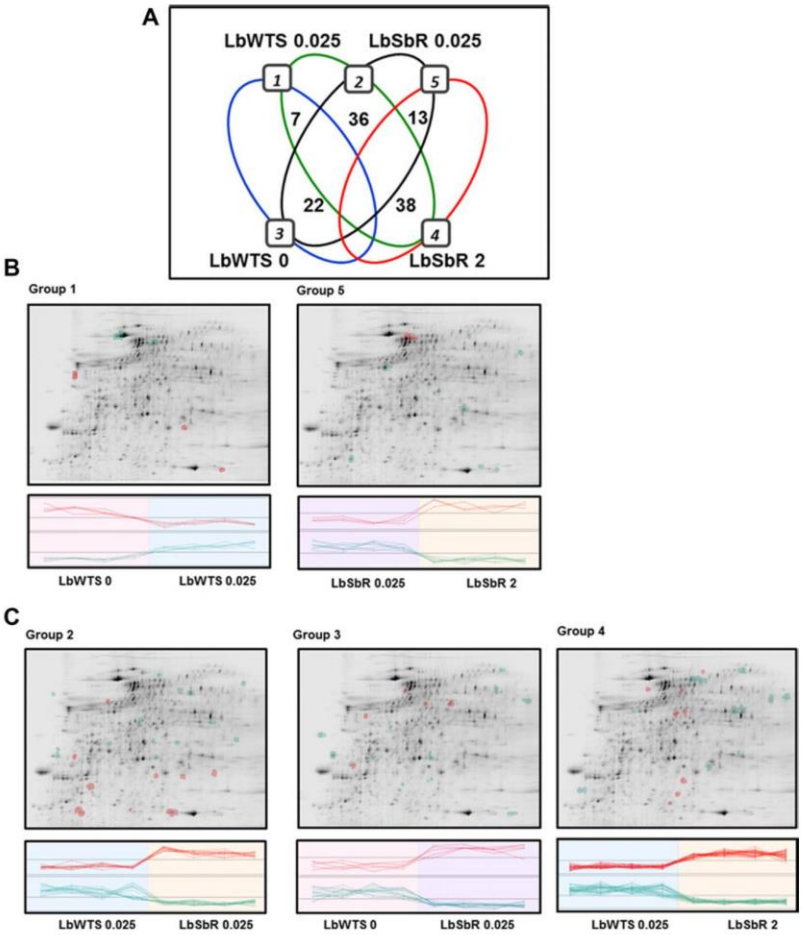

Figure 2. Pair wise comparisons. (A) Venn diagram of the pair wise comparative analysis for groups 1–5. Numbers within squares represent the groups and the numbers in the diagram represent the total number of phosphoproteins identified within each group. (B) Pair wise comparisons for groups 1 and 5 (this informs on what drug treatment changes in terms of phosphorylation and thus informs on drug-responsive signaling). (C) Pair wise comparative analysis for groups 2, 3, and 4 (this informs on potential resistance mechanisms linked to phosphorylation).

Table 1. Analysis of 2D-DIGE phosphoprotein profiles from different groups of *L. braziliensis*

Groups of *L. braziliensis*[a)]	MS/MS[b)]	SS[c)]	TP[d)]	DP[e)]	Table[f)]
1 LbWTS 0 x LbWTS 0.025	9	4	7	4	2
2 LbWTS 0.025 x LbSbR 0.025	81	13	36	28	3
3 LbWTS 0 x LbSbR 0.025	61	7	22	17	4
4 LbWTS 0.025 x LbSbR 2	66	14	38	29	5
5 LbSbR 0.025 x LbSbR 2	29	7	13	10	6

a) Division in groups of the phosphoproteins identified according to the treatment submitted for the antimony-susceptible (WTS) and -resistant (SbR) lines of *L. braziliensis*.
b) Number of spots analyzed by MS/MS that showed a minimum of 1.5-fold change in fluorescence intensity with a p-value < 0.05 (ANOVA).
c) Number of specific spots in the group analyzed.
d) Total number of phosphoproteins identified in the group analyzed.
e) Number of different phosphoproteins identified in the group analyzed.
f) Number of the table where the phosphoproteins identified are shown.

3.3 Phosphoproteins specifically associated to the response induced by SbIII

The groups 1 and 5 comparisons (Tables 2 and 6, Fig. 2B) reveal the parasite signaling response to the stress induced by the drug. Some of the differentially abundant spots were only observed in these two groups and are therefore exclusively linked to the response to SbIII treatment.

The differences observed in phosphoprotein abundance in response to the drug signal are narrow with fold changes below 2 despite significant p-values < 0.05 (Tables 2 and 6). However, as expected, the main biological process category targeted in response to SbIII is "protein folding/chaperones and stress response," including heat shock proteins HSP70 (LbrM.28.2990) and HSP83-1 (LbrM.33.0340). In general, the heat shock response is a homeostatic mechanism that protects cells from the deleterious effects of environmental stress, such as heat [36] and drug exposure. One spot corresponding to HSP70 (LbrM.28.2990) was identified specifically when the susceptible parasites were treated with SbIII

Table 2. Phosphoproteins differentially abundant in the group 1 (LbWTS 0 x LbWTS 0.025) of. *L. braziliensis*

Spot no.	LbWTS 0.025 /LbWTS 0[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b)]	Protein score[c)]	p*I*[d)]	MW[e)]	Max CV (%)[f)]	ANOVA (p)[g)]
Protein folding/chaperones and stress response[h)]									
305	1.5	LbrM.28.2990	Heat-shock protein hsp70, putative	14	627	5.22/5.49	71 277/70 676	11.2	4.9E-04
1528	1.7	LbrM.33.0340	Heat shock protein 83-1	15	274	4.8/5.09	80 663/79 324	17.5	3.3E-03
1530	1.7	LbrM.33.0340	Heat shock protein 83-1	14	222	4.8/5.07	80 663/79 865	11.2	3.4E-04
1529	1.6	LbrM.33.0340	Heat shock protein 83-1	11	244	4.8/5.03	80 663/79 189	7	4.0E-05
Spot no.	LbWTS 0 / LbWTS 0.025[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b)]	Protein score[c)]	p*I*[d)]	MW[e)]	Max CV (%)[f)]	ANOVA (p)[g)]
Protein folding/chaperones and stress response[h)]									
613	1.5	LbrM.31.2940	Calreticulin, putative	5	178	4.1/4.54	48 473/46 273	22.3	1.1E-02
650	1.5	LbrM.31.2940	Calreticulin, putative	5	255	4.1/4.54	48 473/43 091	13	1.8E-03
Antioxidant/detoxification									
1506	1.7	LbrM.34.2140	Kinetoplastid membrane protein-11	3	164	6.52/6.3	11 170/12 160	16.3	3.9E-03

a) Ratio of more abundance of protein spots between the samples LbWTS 0.025 and LbWTS 0 or LbWTS 0 and LbWTS 0.025.
b) Number of peptides identified.
c) MASCOT score.
d) Isoelectric point — theoretical/experimental.
e) Molecular weight (Da) — predicted/experimental.
f) Maximum coefficient of variation (%).
g) The differences are statistically significant for all spots presented in the table ($p < 0.05$).
h) Biological process categories according to GO and KEGG orthology databases.
The underlined spot numbers are specific to a given comparison.

and compared to LbWTS 0 (spot 305, Table 2). Likewise, increase abundance of HSP83-1 (LbrM.33.0340) proteins was specifically related to SbIII treatment as revealed by the comparison of group 1 (spots 1528, 1529, and 1530) and group 5 (spots 262, 264, and 268) (Tables 2 and 6). These results demonstrate that the stress caused by the treatment of the LbWTS line with 0.025 mg/mL of SbIII and LbSbR with 2 mg/mL leads to increased phosphorylation of the major parasite HSPs supporting the hypothesis that *Leishmania* stress proteins are regulated on posttranslational levels by phosphorylation [28].

On the other hand, our results also demonstrated that one spot identified as protein disulfide isomerase (LbrM.35.7320), a thioredox chaperone that has been reported to be overexpressed in a SbV-resistant line of *L. panamensis* [37], and spots corresponding to prolyl oligopeptidase (LbrM.35.7100), rieske iron-sulfur protein precursor (LbrM.34.1450), and one hypothetical protein (LbrM.28.2090) were more abundant in LbSbR 0.025 compared to LbSbR 2 (Table 6). These results show that the stress caused by the high SbIII concentration (2 mg/mL) decreases the abundance of these phosphoproteins in the sample LbSbR 2.

3.4 Phosphoproteins specifically associated to resistance mechanisms against SbIII

The group 2, 3, and 4 comparisons (Tables 3–5) may inform on resistance mechanisms. Some of the spots differentially abundant were only observed in these three groups and could be exclusively linked to the SbIII resistance.

3.4.1 Protein folding/chaperones and stress response

The proteins related to this biological process were found increased mainly in the susceptible parasites. Thus, the spots 338 and 375 corresponding to HSP70 (LbrM.28.2990) identified only in the group 2 were more abundant in LbWTS 0.025 than in LbSbR 0.025, suggesting that some isoforms or modifications of this protein are specifically related to drug response or drug resistance mechanisms (Table 3). Our study also revealed spots corresponding to a tetratricopeptide repeat (TPR) domain protein (LbrM.30.2700), which may act as a scaffold for multiprotein complexes involved in a variety of cellular functions (Tables 3–5). Spots corresponding to calreticulin (LbrM.31.2940) were found in groups 1, 2, 4, and 5. They are not specific to the stress response or the resistance mechanisms, and will be discussed later with other proteins.

3.4.2 Antioxidant/detoxification

The major biological process implicated in resistance mechanisms is the antioxidant/detoxification process, with the exception of kinetoplastid membrane protein-11

Table 3. Phosphoproteins differentially abundant in the group 2 (LbWTS 0.025 x LbSbR 0.025) of *L. braziliensis*

Spot no.	LbSbR 0.025 / LbWTS 0.025[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. Count[b)]	Protein Score[c)]	pI[d)]	MW[e)]	Max CV (%)[f)]	ANOVA (p)[g)]
Protein folding/chaperones and stress response[h)]									
650	2.1	LbrM.31.2940	Calreticulin, putative	5	255	4.1/4.54	48 473/43 091	9.6	1.0E-05
Antioxidant/detoxification									
1531	5.9	LbrM.23.0300	Pteridine reductase 1	3	218	5.3/5.9	30 492/32 748	10.9	1.3E-07
1532	4.4	LbrM.23.0300	Pteridine reductase 1	5	263	5.3/5.9	30 492/33 511	12.1	1.3E-06
1533	3.4	LbrM.23.0050	Peroxidoxin	6	302	7.83/5.86	25 388/23 321	19.8	5.1E-05
1479	1.7	LbrM.34.2140	Kinetoplastid membrane protein-11	2	63	6.52/5.73	11 170/12 811	21	4.4E-03
1506	1.7	LbrM.34.2140	Kinetoplastid membrane protein-11	3	164	6.52/6.3	11 170/12 160	16.3	6.7E-04
1150	1.5	LbrM.15.1100	Tryparedoxin peroxidase	5	222	6.51/6.2	22 573/24 275	10.1	7.0E-04
Metabolic process									
1543	2.6	LbrM.32.3210	Nucleoside diphosphate kinase b	4	125	8.06/4.85	16 689/17 308	20.4	2.3E-04
RNA/DNA processing									
1238	1.8	LbrM.13.1240	Ran-binding protein 1, putative	7	295	5.42/5.64	17 850/20 458	22	2.2E-03
793	1.6	LbrM.15.1440	Proliferative cell nuclear antigen (PCNA), putative	8	581	5.42/4.84	37 700/36 679	18.2	4.6E-03
Protein biosynthesis									
558	3.2	LbrM.23.0290	Argininosuccinate synthase, putative	10	351	9.93/5.6	48 559/50 000	10.4	3.5E-06
1237	1.7	LbrM.16.0150	Eukaryotic translation initiation factor 1A, putative	5	264	4.4/4.53	18 709/20 229	14.8	1.2E-03
1326	1.7	LbrM.13.0390	40S ribosomal protein S12, putative	3	139	4.56/4.84	15 631/17 811	21.1	3.4E-03
Hypothetical proteins									
1302	2.1	LbrM.34.4450	Hypothetical protein, conserved	4	119	4.0/4.67	21 809/18 195	13.4	1.6E-04
1544	1.8	LbrM.34.4450	Hypothetical protein, conserved	4	103	4.0/4.87	21 809/17 101	14.8	5.7E-04
1469	2	LbrM.33.0650	Hypothetical protein, conserved	3	116	4.34/4.58	14 425/13 107	15.4	1.8E-04
1460	1.7	LbrM.13.0270	Hypothetical protein, conserved	3	139	5.76/5.26	13 197/13 580	6.6	1.9E-05
1454	1.6	LbrM.35.1650	Hypothetical protein, conserved	2	131	5.1/4.68	19 205/13 728	13.6	3.8E-04
Spot no.	LbWTS 0.025 / LbSbR 0.025[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. Count[b)]	Protein Score[c)]	pI[d)]	MW[e)]	Max CV (%)[f)]	Anova (p)[g)]
Protein folding/chaperones and stress response[h)]									
375	1.7	LbrM.28.2990	Heat-shock protein hsp70, putative	11	785	5.22/5.95	71 277/65 091	23.9	8.0E-03
338	1.5	LbrM.28.2990	Heat-shock protein hsp70, putative	8	64	5.22/5.82	71 277/67 818	21.8	1.3E-02
686	1.7	LbrM.30.2700	TPR domain protein, conserved	9	311	4.87/5.47	45 655/40 818	10.1	2.3E-04
652	1.5	LbrM.30.2700	TPR domain protein, conserved	6	97	4.87/5.32	45 655/42 909	22.9	1.3E-02
Metabolic process									
886	2	LbrM.35.3170	Succinyl-CoA ligase [GDP-forming] beta-chain, putative	4	227	7.04/5.55	43 906/34 046	25.6	1.2E-02
1545	1.4	LbrM.35.3170	Succinyl-CoA ligase [GDP-forming] beta-chain, putative	4	118	7.04/5.8	43 906/34 427	16.3	1.3E-02
267	1.7	LbrM.33.0320	ATP-binding cassette sub-family F member 1, putative	10	175	7.02/6.54	74 199/74 459	13.9	8.6E-04
172	1.6	LbrM.35.1490	Transitional endoplasmic reticulum ATPase, putative	13	439	5.05/5.4	87 287/87 973	11.8	5.9E-04
188	1.6	LbrM.35.1490	Transitional endoplasmic reticulum ATPase, putative	10	128	5.05/5.59	87 287/85 135	15	2.1E-03
RNA/DNA processing									
383	1.9	LbrM.30.3340	UV excision repair RAD23-like protein	4	178	4.07/4.4	44 971/64 727	12.4	2.0E-04
Protein biosynthesis									
154	2.1	LbrM.35.0270	Elongation factor 2	19	432	5.97/6.28	94 249/89 324	22.4	9.4E-04
153	1.9	LbrM.35.0270	Elongation factor 2	14	355	5.97/6.1	94 249/89 459	14.7	3.9E-04

Table 3. Continued

Spot no.	LbWTS 0.025 / LbSbR 0.025[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. Count[b)]	Protein Score[c)]	pI[d)]	MW[e)]	Max CV (%)[f)]	Anova (p)[g)]
603	1.5	LbrM.01.0740	Eukaryotic initiation factor 4a, putative	8	507	6.15/6.0	45 327/47 000	6.7	4.3E-05
341	1.6	LbrM.15.0260	Lysyl-tRNA synthetase, putative	10	135	6.8/6.66	67 052/67 545	7.5	2.2E-05
Hypothetical proteins									
869	2.3	LbrM.14.0460	Hypothetical protein, conserved	3	156	4.73/4.83	36 668/34 504	9.1	1.2E-05
1075	1.9	LbrM.30.3080	Hypothetical protein, conserved	3	88	6.9/4.51	36 367/27 214	22.7	1.8E-03
1012	1.7	LbrM.25.1690	Hypothetical protein, conserved	3	138	4.19/4.44	23 677/29 695	19	6.6E-03
682	1.6	LbrM.30.0640	Hypothetical protein, conserved	15	618	5.3/5.53	36 852/41 273	13.9	1.1E-03

a) Ratio of more abundance of protein spots between the samples LbSbR 0.025 and LbWTS 0.025 or LbWTS 0.025 and LbSbR 0.025.
b) Number of peptides identified.
c) MASCOT score.
d) Isoelectric point — theoretical/experimental.
e) Molecular weight (Da) — predicted/experimental.
f) Maximum coefficient of variation (%).
g) The differences are statistically significant for all spots presented in the table ($p < 0.05$).
h) Biological process categories according to GO and KEGG orthology databases.
The underlined spot numbers are specific to a given comparison.

(KMP-11) (LbrM.34.2140) and pteridine reductase 1 (PTR1) (LbrM.23.0300) that were identified in almost all groups and will be discussed later. Spots corresponding to peroxidoxin (LbrM.23.0050) and tryparedoxin peroxidase (LbrM.15.1100) were identified with increased abundance in the LbSbR 0.025 compared to the LbWTS 0.025 (Table 3 and Supporting Information Fig. 6). Peroxidoxins comprise a family of antioxidants that have been recently discovered in numerous prokaryotes and eukaryotes and play key roles in the defence against oxidative stress. The expression level of peroxiredoxin was found increased in an antimony-resistant line of *L. infantum* [24]. Tryparedoxin peroxidase is a member of the peroxiredoxin family and a key enzyme that catalyzes the terminal reaction of a cascade of enzymes required for peroxides detoxification [38, 39]. Previous studies have associated high levels of tryparedoxin peroxidase with resistance to arsenite and antimony in resistant mutants of *Leishmania* [40, 41]. Indeed, increased expression of this protein was observed in antimony unresponsive *L. donovani* field isolates [42], in a gentamicin-resistant line of *L. infantum* [43], in SbIII-resistant lines of *L. braziliensis* and *L. infantum* [24], and in SbIII-resistant *L. infantum* mutant [44].

3.4.3 Cytoskeletal proteins

Various cytoskeletal proteins showed a 1.6 to 2.6-fold decreased in abundance in LbSbR 2 (group 4). Our study revealed protein spots corresponding to alpha-tubulin (LbrM.13.0190) and beta-tubulin (LbrM.33.1010) (Table 5). Tubulins are known to undergo numerous PTMs, such as acetylation, phosphorylation, polyglutamylation, polyglycylation, detyrosination, among others [45]. Previous studies have reported that the phosphorylation of tubulins could affect the dynamics of tubulin assembly or could be indicative of their roles in several signal transduction pathways [46, 47]. The alpha- and beta-tubulin proteins have been described as the basic components of microtubules, which are crucial for the mitotic spindle apparatus during cell division, intracellular transport and ciliary and flagellar motility [48]. Beta-tubulin has been identified upregulated in arsenite-resistant *L. donovani* [49], in methotrexate-resistant *L. major* [21], in antimony-resistant isolates of *L. donovani* [50, 51], in gentamicin-resistant *L. infantum* line [43], and in SbIII-resistant line of *L. braziliensis* [24]. Christen et al. [52] suggested that the sensitivity of cancer cells to chemotherapeutic agents is mediated by phosphorylation of beta-tubulin, establishing the possible role of tubulin in the signal transduction cascades regulating drug sensitivity and, therefore, resistance. Other cytoskeletal proteins were also observed in our comparative analysis like the paraflagellar rod protein 1D (LbrM.31.0160) identified in two spots (Table 5). Paraflagellar rod protein 1D was observed downregulated in the antimony-resistant *L. braziliensis* line [24]. These results reveal that the paraflagellar rod protein 1D shows lower abundance in the phosphoproteome as well as the total proteome in SbIII-resistant *L. braziliensis*.

3.4.4 Metabolic process

Three proteins associated with metabolic processes were identified and specifically more abundant in the phosphoproteome of resistant parasites when compared to susceptible ones. The metallo-peptidase, Clan MA(E), Family M32 (LbrM.33.2810), an enzyme involved in proteolysis (Table 4), the acidocalcisomal pyrophosphatase (LbrM.19.0110) (Table 5) and the protein nucleoside diphosphate kinase b (NDKb) (LbrM.32.3210). This latter enzyme catalyzes the exchange of phosphate groups between different nucleoside diphosphates and was identified in two spots within groups 2, 3, and 4 (spots 1333 and 1543, Tables 3–5 and Supporting Information Fig. 6). As NDKb has been found unchanged or downregulated in different antimony-resistant

Table 4. Phosphoproteins differentially abundant in the group 3 (LbWTS 0 x LbSbR 0.025) of *L. braziliensis*

Spot no.	LbSbR 0.025 / LbWTS 0[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b)]	Protein score[c)]	pI[d)]	MW[e)]	Max CV (%)[f)]	ANOVA (p)[g)]
Antioxidant/detoxification[h)]									
1531	4.6	LbrM.23.0300	Pteridine reductase 1	3	218	5.3/5.9	30 492/32 748	18.3	3.9E-06
1532	3.7	LbrM.23.0300	Pteridine reductase 1	5	263	5.3/5.9	30 492/33 511	14.9	5.7E-06
Metabolic process									
1543	2.3	LbrM.32.3210	Nucleoside diphosphate kinase b	4	125	8.06/4.85	16 689/17 308	26.1	6.5E-04
482	1.6	LbrM.33.2810	Metallo-peptidase, Clan MA(E), Family M32	14	608	5.52/5.47	57 206/56 636	13.1	3.6E-03
RNA/DNA processing									
466	1.6	LbrM.31.1980	Nucleosome assembly protein-like protein	6	179	4.2/4.49	45 551/57 545	7.2	3.2E-05
793	1.6	LbrM.15.1440	Proliferative cell nuclear antigen (PCNA), putative	8	581	5.42/4.84	37 700/36 679	11.8	8.1E-04
Protein biosynthesis									
558	4.4	LbrM.23.0290	Argininosuccinate synthase, putative	10	351	9.93/5.6	48 559/50 000	32.3	1.3E-04
1326	1.7	LbrM.13.0390	40S ribosomal protein S12, putative	3	139	4.56/4.84	15 631/17 811	15.7	1.4E-03
Hypothetical proteins									
1302	2	LbrM.34.4450	Hypothetical protein, conserved	4	119	4.0/4.67	21 809/18 195	13.4	1.7E-04
1544	1.7	LbrM.34.4450	Hypothetical protein, conserved	4	103	4.0/4.87	21 809/17 101	10.2	2.8E-04
1469	1.8	LbrM.33.0650	Hypothetical protein, conserved	3	116	4.34/4.58	14 425/13 107	9.4	5.3E-05
597	1.5	LbrM.29.0260	Hypothetical protein, conserved	2	185	4.79/4.78	40 934/47 000	13	1.3E-03

Spot no.	LbWTS 0 / LbSbR 0.025[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b)]	Protein score[c)]	pI[d)]	MW[e)]	Max CV (%)[f)]	ANOVA (p)[g)]
Protein folding/chaperones and stress response[h)]									
652	1.5	LbrM.30.2700	TPR domain protein, conserved	6	97	4.87/5.32	45 655/42 909	9.5	3.7E-04
Metabolic process									
1030	1.9	LbrM.35.3430	14-3-3 protein-like protein	3	211	4.57/4.87	29 690/28 931	23.8	8.9E-03
Protein biosynthesis									
153	2.1	LbrM.35.0270	Elongation factor 2	14	355	5.97/6.1	94 249/89 459	11.3	3.7E-05
150	2.1	LbrM.35.0270	Elongation factor 2	20	566	5.97/6.36	94 249/89 595	19.8	4.7E-04
154	1.9	LbrM.35.0270	Elongation factor 2	19	432	5.97/6.28	94 249/89 324	10.4	5.1E-05
603	1.9	LbrM.01.0740	Eukaryotic initiation factor 4a, putative	8	507	6.15/6.0	45 327/47 000	13	1.9E-04
Hypothetical proteins									
869	2.8	LbrM.14.0460	Hypothetical protein, conserved	3	156	4.73/4.83	36 668/34 504	20.6	1.2E-04
1017	2	LbrM.25.1690	Hypothetical protein, conserved	3	391	4.19/4.38	23 677/29 618	15.5	2.7E-04
1014	1.5	LbrM.25.1690	Hypothetical protein, conserved	3	405	4.19/4.41	23 677/29 695	16.9	3.4E-03
1206	1.7	LbrM.02.0520	Hypothetical protein, unknown function	4	240	5.63/5.55	17 529/21 756	13.6	1.2E-03

a) Ratio of more abundance of protein spots between the samples LbSbR 0.025 and LbWTS 0 or LbWTS 0 and LbSbR 0.025.
b) Number of peptides identified.
c) MASCOT score.
d) Isoelectric point — theoretical/experimental.
e) Molecular weight (Da) — predicted/experimental.
f) Maximum coefficient of variation (%).
g) The differences are statistically significant for all spots presented in the table ($p < 0.05$).
h) Biological process categories according to GO and KEGG orthology databases.
The underlined spot numbers are specific to a given comparison.

isolates of *L. donovani* [51] the increase of this protein in the parasite phosphoproteome may indicate an increase in its phosphorylation stoichiometry in resistant lines.

On the other hand, we observed some proteins with increased abundance in the susceptible parasites compared to the resistant ones. Some are involved in the carbohydrate metabolism like the succinyl-CoA ligase [GDP-forming] beta-chain (LbrM.35.3170), that catalyzes the reversible reaction of succinyl-CoA to succinate (Tables 3 and 5). Reduction of this protein was reported in the SbIII-resistant line of *L. infantum* [24]. The spot corresponding to pyruvate kinase (LbrM.34.0010), an enzyme involved in glycolysis, was identified in the group 4 (Table 5). Reduction of this protein was also observed in antimony-resistant axenic amastigote cells of *L. infantum* [53]. Our analysis also identified some proteins involved in the amino acid metabolism and proteolysis that showed differential abundance among the samples analyzed, like the proteasome alpha 1 subunit (LbrM.34.4810), which

Table 5. Phosphoproteins differentially abundant in the group 4 (LbWTS 0.025 x LbSbR 2) of *L. braziliensis*

Spot no.	LbSbR 2/LbWTS 0.025[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b)]	Protein score[c)]	pI[d)]	MW[e)]	Max CV (%)[f)]	ANOVA (p)[g)]
Protein folding/chaperones and stress response[h)]									
613	1.5	LbrM.31.2940	Calreticulin, putative	5	178	4.1/4.54	48 473/46 273	19.8	1.3E-02
Antioxidant/detoxification									
1531	3.6	LbrM.23.0300	Pteridine reductase 1	3	218	5.3/5.9	30 492/32 748	15	9.6E-06
1532	2.9	LbrM.23.0300	Pteridine reductase 1	5	263	5.3/5.9	30 492/33 511	15.5	3.0E-05
Metabolic process									
1543	2.4	LbrM.32.3210	Nucleoside diphosphate kinase b	4	125	8.06/4.85	16 689/17 308	20.4	3.6E-04
1333	1.6	LbrM.32.3210	Nucleoside diphosphate kinase b	5	177	8.06/5.45	16 689/17 485	20.8	7.8E-03
574	1.6	LbrM.19.0110	Acidocalcisomal pyrophosphatase	8	307	5.52/5.44	51 388/49 545	7.8	1.5E-04
RNA/DNA processing									
466	1.7	LbrM.31.1980	Nucleosome assembly protein-like protein	6	179	4.2/4.49	45 551/57 545	14.7	5.0E-04
793	1.5	LbrM.15.1440	Proliferative cell nuclear antigen (PCNA), putative	8	581	5.42/4.84	37 700/36 679	18.2	1.1E-02
1238	1.5	LbrM.13.1240	Ran-binding protein 1, putative	7	295	5.42/5.64	17 850/20 458	10.8	1.6E-03
Protein biosynthesis									
558	3.4	LbrM.23.0290	Argininosuccinate synthase, putative	10	351	9.93/5.6	48 559/50 000	9.7	1.9E-06
1237	1.7	LbrM.16.0150	Eukaryotic translation initiation factor 1A, putative	5	264	4.4/4.53	18 709/20 229	13.1	8.1E-04
1326	1.7	LbrM.13.0390	40S ribosomal protein S12, putative	3	139	4.56/4.84	15 631/17 811	21.1	3.1E-03
Hypothetical proteins									
1302	2.1	LbrM.34.4450	Hypothetical protein, conserved	4	119	4.0/4.67	21 809/18 195	16.1	2.9E-04
1544	1.7	LbrM.34.4450	Hypothetical protein, conserved	4	103	4.0/4.87	21 809/17 101	14.8	1.1E-03
1469	2.3	LbrM.33.0650	Hypothetical protein, conserved	3	116	4.34/4.58	14 425/13 107	15.4	1.2E-04
1215	1.5	LbrM.30.3140	Hypothetical protein, conserved	4	177	5.29/5.51	23 437/21 107	21.2	1.5E-02
Spot no.	LbWTS 0.025 / LbSbR 2[a)]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b)]	Protein score[c)]	pI[d)]	MW[e)]	Max CV (%)[f)]	ANOVA (p)[g)]
Protein folding/chaperones and stress response[h)]									
686	1.8	LbrM.30.2700	TPR domain protein, conserved	9	311	4.87/5.47	45 655/40 818	8.2	4.9E-05
652	1.7	LbrM.30.2700	TPR domain protein, conserved	6	97	4.87/5.32	45 655/42 909	22.9	7.6E-03
Cytoskeletal proteins									
520	2.6	LbrM.13.0190	Alpha tubulin	8	285	4.65/5.0	49 684/53 455	27.7	1.2E-03
243	1.8	LbrM.31.0160	Paraflagellar rod protein 1D	11	185	5.21/5.6	69 263/78 919	17.7	1.1E-03
253	1.7	LbrM.31.0160	Paraflagellar rod protein 1D	7	99	5.21/5.71	69 263/76 081	13.7	5.7E-04
1295	1.6	LbrM.33.1010	Beta-tubulin	4	71	4.38/4.39	20 507/18 373	20.1	1.3E-02
Metabolic process									
1003	2.3	LbrM.34.4810	Proteasome alpha 1 subunit, putative	4	74	6.76/6.14	27 255/30 382	24.4	1.5E-03
886	2.1	LbrM.35.3170	Succinyl-CoA ligase [GDP-forming] beta-chain, putative	4	227	7.04/5.55	43 906/34 046	26	9.7E-03
561	1.8	LbrM.35.4150	S-adenosylhomocysteine hydrolase	8	243	5.94/6.1	47 827/49 909	16.6	9.5E-04
555	1.6	LbrM.35.4150	S-adenosylhomocysteine hydrolase	7	339	5.94/5.99	47 827/50 182	17.6	5.2E-03
443	1.6	LbrM.34.0010	Pyruvate kinase	10	109	6.06/6.51	49 120/60 091	10.8	2.6E-04
RNA/DNA processing									
556	1.7	LbrM.21.1700	RNA helicase, putative	14	264	7.53/6.2	47 152/50 909	13.9	7.4E-04
Protein biosynthesis									
154	3.2	LbrM.35.0270	Elongation factor 2	19	432	5.97/6.28	94 249/89 324	22.4	6.1E-05
1536	3	LbrM.35.0270	Elongation factor 2	13	120	5.97/6.21	94 249/94 851	26.6	4.1E-04
1535	2.5	LbrM.35.0270	Elongation factor 2	17	424	5.97/6.19	94 249/89 865	16.6	1.5E-04
153	2.4	LbrM.35.0270	Elongation factor 2	14	355	5.97/6.1	94 249/89 459	14.7	2.0E-04
603	1.6	LbrM.01.0740	Eukaryotic initiation factor 4a, putative	8	507	6.15/6.0	45 327/47 000	4.5	3.2E-06
341	1.6	LbrM.15.0260	Lysyl-tRNA synthetase, putative	10	135	6.8/6.66	67 052/67 545	8.8	1.5E-04
Hypothetical proteins									
869	2.5	LbrM.14.0460	Hypothetical protein, conserved	3	156	4.73/4.83	36 668/34 504	16.9	5.5E-05
252	1.9	LbrM.32.1870	Hypothetical protein, conserved	3	84	5.78/5.77	55 971/76 216	9.7	2.1E-05

Table 5. Continued

Spot no.	LbWTS 0.025 / LbSbR 2[a]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b]	Protein score[c]	pI[d]	MW[e]	Max CV (%)[f]	ANOVA (p)[g]
1075	1.8	LbrM.30.3080	Hypothetical protein, conserved	3	88	6.9/4.51	36 367/27 214	22.7	9.9E-03
682	1.5	LbrM.30.0640	Hypothetical protein, conserved	15	618	5.3/5.53	36 852/41 273	13.9	2.1E-03

a) Ratio of more abundance of protein spots between the samples LbSbR 2 and LbWTS 0.025 or LbWTS 0.025 and LbSbR 2.
b) Number of peptides identified.
c) MASCOT score.
d) Isoelectric point — theoretical/experimental.
e) Molecular weight (Da) — predicted/experimental.
f) Maximum coefficient of variation (%).
g) The differences are statistically significant for all spots presented in the table ($p < 0.05$).
h) Biological process categories according to GO and KEGG orthology databases.
The underlined spot numbers are specific to a given comparison.

was less abundant in LbSbR 2 (Table 5). The proteasome is a multi-catalytic proteinase complex, which is characterized by its ability to cleave peptides and plays an important role in the regulation of cell cycle control or cell proliferation, stress response, apoptosis, and others. Other spots detected in the groups 2 and 3 were less abundant in the LbSbR 0.025 like the ATP-binding cassette subfamily F member 1 (LbrM.33.0320), the 14-3-3 protein (LbrM35.3430), and the transitional endoplasmic reticulum ATPase (LbrM.35.1490) identified in two spots (Tables 3 and 4). The S-adenosylhomocysteine hydrolase (SAHH) (LbrM.35.4150) identified in this analysis was less abundant in LbSbR 2 (Tables 5 and 6). Although this

Table 6. Phosphoproteins differentially abundant in the group 5 (LbSbR 0.025 x LbSbR 2) of *L. braziliensis*

Spot no.	LbSbR 2 / LbSbR 0.025[a]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b]	Protein score[c]	pI[d]	MW[e]	Max CV (%)[f]	ANOVA (p)[g]
Protein folding/chaperones and stress response[h]									
262	1.6	LbrM.33.0340	Heat shock protein 83-1	16	604	4.8/5.36	80 663/75 135	11.3	1.1E-03
264	1.6	LbrM.33.0340	Heat shock protein 83-1	12	362	4.8/5.4	80 663/75 000	11.1	2.7E-04
268	1.5	LbrM.33.0340	Heat shock protein 83-1	17	633	4.8/5.31	80 663/74 459	12.4	3.8E-03
Spot no.	LbSbR 0.025 / LbSbR 2[a]	TriTrypDB accession no. *L. braziliensis*	Protein identity	Pep. count[b]	Protein score[c]	pI[d]	MW[e]	Max CV (%)[f]	ANOVA (p)[g]
Protein folding/chaperones and stress response[h]									
552	1.7	LbrM.35.7320	Protein disulfide isomerase	7	134	4.77/5.08	52 391/50 727	13.2	6.5E-04
650	1.5	LbrM.31.2940	Calreticulin, putative	5	255	4.1/4.54	48 473/43 091	21.7	1.2E-02
Antioxidant/detoxification									
1506	1.8	LbrM.34.2140	Kinetoplastid membrane protein-11	3	164	6.52/6.3	11 170/12 160	17.4	5.8E-04
1531	1.6	LbrM.23.0300	Pteridine reductase 1	3	218	5.3/5.9	30 492/32 748	15	8.7E-04
1532	1.5	LbrM.23.0300	Pteridine reductase 1	5	263	5.3/5.9	30 492/33 511	15.5	4.6E-03
Metabolic process									
224	1.6	LbrM.35.7100	Prolyl oligopeptidase, putative	10	249	6.05/6.0	78 335/77 973	8	7.8E-05
555	1.6	LbrM.35.4150	S-adenosylhomocysteine hydrolase	7	339	5.94/5.99	47 827/50 182	13	1.6E-03
819	1.5	LbrM.34.1450	Rieske iron-sulfur protein, mitochondrial precursor, putative	7	284	6.52/5.62	33 774/35 992	10.9	6.4E-04
Protein biosynthesis									
154	1.5	LbrM.35.0270	Elongation factor 2	19	432	5.97/6.28	94 249/89 324	10.4	5.5E-04
Hypothetical proteins									
1459	1.5	LbrM.28.2090	Hypothetical protein, conserved	2	92	6.55/6.12	13 107/13 669	11.9	1.8E-03

a) Ratio of more abundance of protein spots between the samples LbSbR 2 and LbSbR 0.025 or LbSbR 0.025 and LbSbR 2.
b) Number of peptides identified.
c) MASCOT score.
d) Isoelectric point — theoretical/experimental.
e) Molecular weight (Da) — predicted/experimental.
f) Maximum coefficient of variation (%).
g) The differences are statistically significant for all spots presented in the table ($p < 0.05$).
h) Biological process categories according to GO and KEGG orthology databases.
The underlined spot numbers are specific to a given comparison.

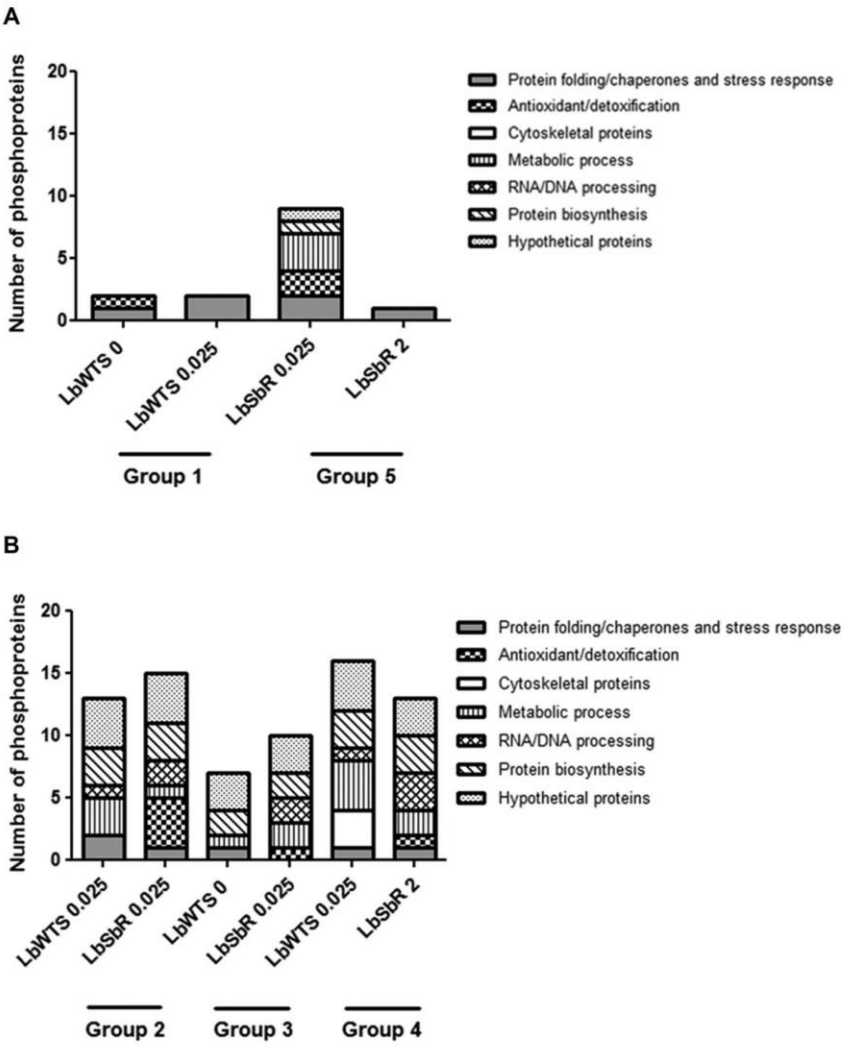

Figure 3. Biological process categories. (A) Bar plot representation of the number of different phosphoproteins increased in each category in response to drug stress for groups 1 and 5. (B) Bar plot representation of the number of different phosphoproteins increased in each category associated to drug resistance phenotype for groups 2, 3, and 4.

protein is less phosphorylated in this sample, some authors have reported that it is more expressed in antimony-resistant isolates of *L. donovani* [51] and in the lines of *L. panamensis* resistant to SbIII [54].

3.4.5 RNA/DNA processing

Three proteins were found more abundant in the resistant parasites (LbSbR 0.025 or LbSbR 2) compared to susceptible ones. First, the proliferative cell nuclear antigen (PCNA) (LbrM.15.1440) (Tables 3–5), an important protein for DNA synthesis and repair in eukaryotic cells that has been reported to be overexpressed in *L. donovani* clinical isolates resistant to SbV [50], and in SbIII-resistant line of *L. braziliensis* [24]. Second, the Ran-binding protein 1 (LbrM.13.1240), which is essential for RNA translocation (Tables 3 and 5). Finally, the nucleosome assembly protein (LbrM.31.1980), which participates in DNA replication and modulation of chromatin (Tables 4 and 5). These results demonstrate that phosphorylation may modulate the activity of important pathways implicated in RNA and DNA processing in SbIII-resistant *L. braziliensis*.

On the other side, we also identified phosphoproteins with lower abundance in the antimony-resistant samples studied, including the UV excision repair RAD23-like protein (LbrM.30.3340) that was identified in group 2 (Table 3) and is known to participate in DNA damage repair. Likewise, an RNA helicase (LbrM.21.1700) was identified in group 4 (Table 5), which may be in RNA duplex unwinding, protein displacement from RNA, or strand annealing [55]. This RNA

helicase showed decreased expression in antimony-resistant *L. infantum* [24].

3.4.6 Protein biosynthesis

We identified three proteins with increased abundance in the resistant parasites and three others with increased abundance in the susceptible ones.

The spot 558 corresponding to argininosuccinate synthase (ASS) (LbrM.23.0290), a key enzyme of the urea cycle that catalyzes the rate-limiting step in the conversion of L-citrulline to L-arginine, was 3.2 to 4.4-fold more abundant in the phosphoproteome of SbIII-resistant parasites (Tables 3–5 and Supporting Information Fig. 6). ASS was found overexpressed in *L. infantum* antimony resistant axenic amastigotes [53]. Eukaryotic translation initiation factor 1A (LbrM.16.0150) was identified in groups 2 and 4 (spot 1237, Tables 3 and 5). Our results also identified spot corresponding to 40S ribosomal protein S12 (LbrM.13.0390), which is involved in ribosomal biogenesis (spot 1326, Table 3–5). In contrast, lysyl-tRNA synthetase (LbrM.15.0260) that catalyzes the formation of lysyl-transfer RNA, and eukaryotic initiation factor 4a (LbrM.01.0740) showed increased abundance in the phosphoproteome of susceptible parasites (LbWTS 0 and LbWTS 0.025) (Tables 3–5). Four different spots corresponding to elongation factor 2 (EF2) (LbrM.35.0270) were identified within almost all the groups (Tables 3–6) and will be discussed later.

3.4.7 Hypothetical proteins

We identified 12 phosphoproteins annotated as hypothetical proteins, which showed differential abundance in the groups 2, 3, and 4 analyzed in this study. Of these hypothetical proteins, LbrM.13.0270, LbrM.29.0260, LbrM.30.3140, and LbrM.35.1650 were specifically observed in one of the groups 2, 3, or 4 and always associated with an increase in abundance for the resistant parasites in comparison with the susceptible ones (Tables 3–5). According to data deposited in GO (http://geneontology.org/) and InterPro (http://www.ebi.ac.uk/interpro/) databases some of these hypothetical proteins are associated with nucleic acid binding (LbrM.13.0270), chaperonin-binding (LbrM.29.0260), and the endoplasmic reticulum (LbrM.30.3140). LbrM.33.0650 (spot 1469) and LbrM.34.4450 (spots 1302 and 1544) were shared among groups 2, 3, and 4 (Tables 3–5), with a domain HSP20-like chaperone, indicating that they can be associated with the antimony resistance phenotype in *L. braziliensis*.

On the other hand, we observed that six hypothetical proteins (LbrM.02.0520, LbrM.14.0460, LbrM.25.1690, LbrM.30.0640, LbrM.30.3080, and LbrM.32.1870) were more abundant in the SbIII-susceptible samples (Tables 3–5) with the LbrM.25.1690 being identified in three spots (1012, 1014, and 1017) specific to groups 2 or 3. According to UniProt (http://www.uniprot.org/), TriTrypDB (http://tritrypdb.org/tritrypdb/), and InterPro (http://www.ebi.ac.uk/interpro/) databases, we observed that the hypothetical protein LbrM.14.0460 has a domain HSP20-like chaperone, and that LbrM.25.1690 and LbrM.30.3080 are implicated in translation initiation factor activity and RNA binding, respectively.

3.5 Phosphoproteins associated to stress and resistance mechanisms against SbIII

Some protein spots identified as calreticulin (LbrM.31.2940), kinetoplastid membrane protein-11 (LbrM.34.2140), pteridine reductase 1 (LbrM.23.0300), and elongation factor 2 (LbrM.35.0270) were found modulated in response to drug treatment and resistance phenotype.

Thus, calreticulin (LbrM.31.2940), a chaperonin that promotes the folding and assembly of synthesized glycoproteins, was identified in two spots (613 and 650) across the groups 1, 2, 4, and 5. In response to SbIII, this protein is decreased in abundance in the LbWTS 0.025 and in the LbSbR 2 (Tables 2 and 6), whereas it is increased in abundance in the SbIII-resistant parasites (LbSbR 0.025 and LbSbR 2) compared to LbWTS 0.025 (Tables 3 and 5). This suggests that an increase in phosphorylated calreticulin may be important in the mechanisms of drug resistance, while a decrease is related to the stress response induced by the drug.

Our results showed that two spots corresponding to kinetoplastid membrane protein-11 (KMP-11) (LbrM.34.2140) were identified in the groups 1, 2, and 5 with one spot (1479) specifically increased in LbSbR 0.025 compared to LbWTS 0.025, indicating a role of this increased form of KMP-11 in SbIII resistance mechanisms (Table 3). Even though KMP-11 is more phosphorylated in this Sb-resistant line, some authors observed that this protein is less expressed in SbIII-resistant axenic amastigote of *L. infantum* [53]. The second spot (1506) is increased in LbWTS 0 compared to LbWTS 0.025 and in LbSbR 0.025 than in LbSbR 2, suggesting that a decrease in abundance of KMP-11 could be linked to stress response to antimony (Tables 2 and 6).

Pteridine reductase 1 (PTR1) (LbrM.23.0300) was identified in two spots (1531 and 1532) in groups 2, 3, 4, and 5 (Tables 3–6 and Supporting Information Fig. 6). This protein was found highly abundant in the resistant parasites with fold changes from 2.9 to 5.9 for the two spots indicating that it can be regulated posttranslationally by phosphorylation (Tables 3–5). However, PTR1 was more abundant in LbSbR 0.025 compared to LbSbR 2, suggesting that the response of resistant parasites to high dose of SbIII could be linked to the decrease in abundance of PTR1 (Table 6). This NADPH-dependent enzyme that reduces pteridines such as biopterin and folate, which are essential for the growth of *Leishmania* [56], was upregulated in methotrexate-resistant mutant of *L. major* [21], and in SbIII-resistant *L. braziliensis* line [24]. These data demonstrate that this protein is more abundant in the

total proteome as well as the phosphoproteome of resistant parasites analyzed in this study.

We observed five spots corresponding to elongation factor 2 (EF2) (LbrM.35.0270), which promotes the GTP-dependent translocation of the nascent protein chain from the aminoacil-site to the peptidil-site of the ribosome (Tables 3–6 and Supporting Information Fig. 6). Three spots (150, 1535, and 1536) were specifically found in groups 3 or 4 (Tables 4 and 5). The increase abundance was observed in the LbWTS parasites compared to LbSbR (Tables 3–5) with the exception of one spot (154) more abundant in LbSbR 0.025 compared to LbSbR 2 (Table 6). Recent data demonstrated that this protein was overexpressed in SbV-resistant isolates of *L. donovani* [51], in SbIII-resistant promastigotes lines of *L. panamensis* [54], and in antimony-resistant *L. infantum* line [24]. Therefore, decrease in abundance of EF2 in our analysis could be implicated in mechanisms of drug resistance but also in the response to high drug pressure. EF2 is regulated by phosphorylation and dephosphorylation by a specific kinase known as eEF2 kinase, which itself is upregulated by various mechanisms in the eukaryotic cell [57].

3.6 In silico analysis

In this study, we used the phosphoresidues sequences previously reported in *Leishmania* species [29–31] to investigate whether the positions of the phosphorylation sites (p-sites) were conserved in some phosphoproteins identified in our analysis. The complete sequences for alignment were obtained from TriTrypDB (http://tritrypdb.org/tritrypdb/). After to perform multiple sequence alignments using ClustalW2 (http://tritrypdb.org/tritrypdb/), the conservation of the p-sites between *L. braziliensis* and other trypanosomatids was evaluated. The results demonstrated that nine proteins identified in our study may be phosphorylated on S^{351} (HSP70), S^{529} (HSP83-1), S^{171} (peroxidoxin), S^{216} (alpha-tubulin), S^{140} and S^{145} (nucleoside diphosphate kinase b), T^{135} (eukaryotic initiation factor 4a), S^{38} (elongation factor 2), S^{186} (hypothetical protein - LbrM.25.1690), and S^{48} (hypothetical protein - LbrM.34.4450) (Fig. 4). We observed that p-sites presented high degree of conservation among the *Leishmania* species for these phosphoproteins analyzed. Interestingly, the results also showed the conservation of the phosphorylated residues for the proteins like nucleoside diphosphate kinase b, eukaryotic initiation factor 4a, elongation factor 2, and LbrM.34.4450 in the sequences of all parasites species investigated (Fig. 4).

3.7 Analysis of the expression and phosphorylation levels of phosphoproteins in SbIII treated and nontreated *L. braziliensis*

We performed Western blot analysis to evaluate the expression and phosphorylation levels of cytosolic tryparedoxin peroxidase (cTXNPx), kinetoplastid membrane protein-11 (KMP-11), and alpha-tubulin in the different samples of *L. braziliensis* analyzed in this study. Our phosphoproteomic analysis showed that the protein spot corresponding to TXNPx was 1.5-fold more abundant in LbSbR 0.025 compared to LbWTS 0.025 (Table 3). Western blot analysis using a polyclonal antibody against cTXNPx of *T. cruzi* [35] showed that this antibody recognized a band of approximately 23 kDa as expected, presenting intensity 1.5-fold higher in the phospho-extract LbSbR 0.025 than in the LbWTS 0.025 (Fig. 5C). The identity between the cTXNPx amino acid sequences of *T. cruzi* compared to *L. braziliensis* correspond to 69%. Interestingly, we can observe that the level of the cTXNPx phosphoprotein was also increased 1.5-fold in LbSbR 2 than in LbWTS 0.025. As previously reported, we also noted that this protein showed a twofold higher abundance in LbSbR 2 (total protein extract) compared to its respective susceptible counterpart LbWTS 0 (Fig. 5C) [24]. This result indicates that the expression and phosphorylation levels of cTXNPx are increased in SbIII-resistant *L. braziliensis* samples.

Analysis of the *L. braziliensis* phosphoproteome demonstrated that the KMP-11 protein showed an abundance of 1.7-fold lower in LbWTS 0.025 compared to LbWTS 0 (Table 2). This protein was also found 1.7-fold more abundant in LbSbR 0.025 than in LbWTS 0.025 (Table 3). Moreover, KMP-11 was decreased 1.8-fold in LbSbR 2 in comparison with LbSbR 0.025 (Table 6). Western blot analysis using a polyclonal antibody *L. braziliensis* anti-KMP-11 showed that this antibody recognized an 11 kDa band in all samples analyzed. This band was 1.5-fold less abundant in phospho-extract LbWTS 0.025 than in LbWTS 0, demonstrating that the treatment of antimony-susceptible line of *L. braziliensis* with SbIII 0.025 mg/mL decreases the phosphorylation level of the KMP-11 protein in LbWTS 0.025 (Fig. 5D). Furthermore, we also observed that the phosphorylation level of this protein was 1.7-fold higher in LbSbR 0.025 compared to LbWTS 0.025 (Fig. 5D). However, no difference in the phosphorylation level was detected between the phospho-extracts LbSbR 0.025 and LbSbR 2 (Fig. 5D). Besides, we did not observe difference among the total protein extracts (Fig. 5D).

Comparative phosphoproteomic analysis between LbWTS 0.025 and LbSbR 2 identified protein spot corresponding to alpha-tubulin, which was 2.6-fold less abundant in LbSbR 2 (Table 5). Western blot results using a monoclonal anti-alpha-tubulin antibody produced in mouse (Sigma) showed that this antibody recognized a band of approximately 50 kDa with abundance 2.8-fold lower in phospho-extract LbSbR 2 compared to LbWTS 0.025 (Fig. 5E). It is important to note that the identity of alpha-tubulin amino acid sequences between mammalian and *L. braziliensis* is 82%. Furthermore, we can observe that phosphorylation level of alpha-tubulin is twofold higher in LbWTS 0 in relation to LbWTS 0.025, and 3.5-fold more abundant in LbSbR 0.025 than in LbSbR 2 (Fig. 5E). This evidences that the treatment of the LbWTS and LbSbR lines with 0.025 and 2 mg/mL of SbIII reduces the phosphorylation level of the alpha-tubulin in LbWTS 0.025

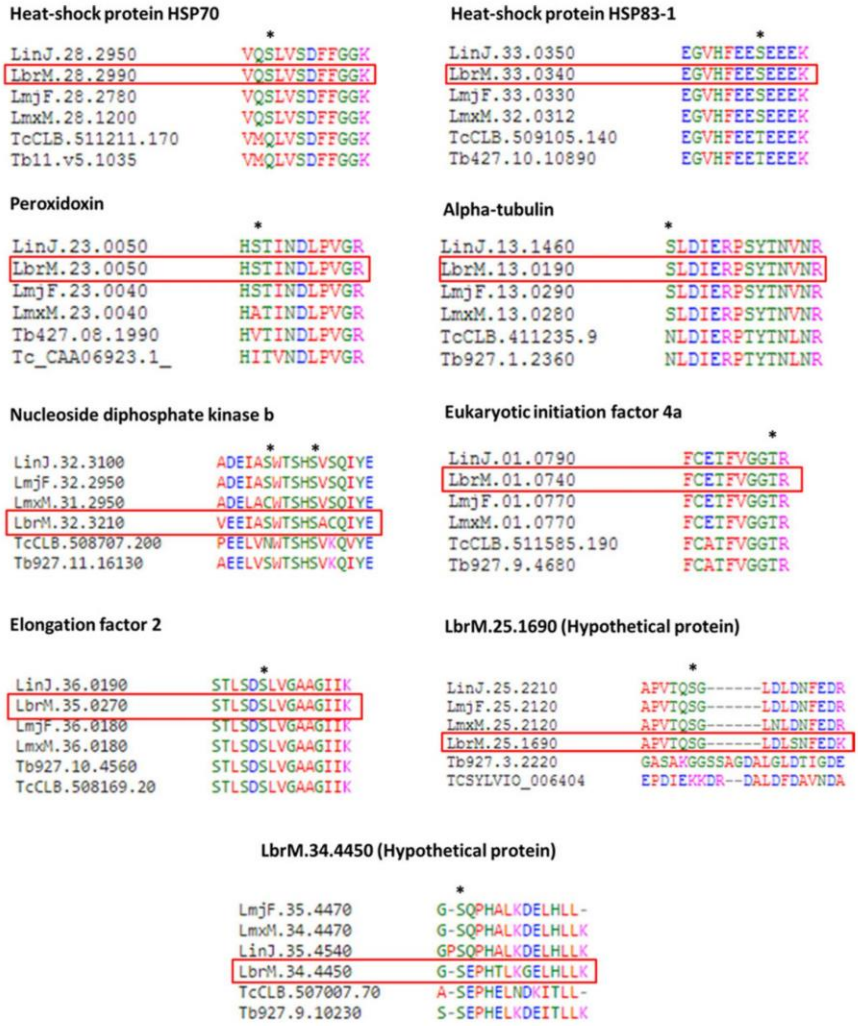

Figure 4. Multiple sequence alignments of phosphopeptides with orthologs from other trypanosomatids. The phosphorylated peptide sequences identified by Hem et al. [29] and Tsigankov et al. [30, 31] were used as reference for alignment and identification of possible p-sites of some phosphoproteins observed in our study. The phosphoresidues are marked by the asterisk (*) and the sequences in red boxes are related to our study. Linf, *L. infantum*; Lbr, *L. braziliensis*; Lmj, *L. major*; Lmx, *L. mexicana*; Tc, *T. cruzi*; Tb, *T. brucei*. ClustalW2 was used for alignment of the sequences.

and LbSbR 2. Furthermore, no difference in the expression level of this protein was observed in the control with total protein extracts (Fig. 5E).

4 Discussion

The posttranslational regulation of a variety of intracellular events through the reversible phosphorylation and dephosphorylation of proteins plays a key role in the biology of trypanosomatids. Although the *Leishmania* phosphoproteome has not been analyzed to completion, it is estimated that as much as one third of the eukaryotic proteome is phosphorylated [58]. Complex networks of protein phosphorylation are modulated by protein kinases and phosphatases, several of which have been identified in trypanosomatids [59, 60]. Thus, the use of phosphoproteomics techniques allows elucidating the signaling pathways in these parasites through the quantification of changes in phosphorylation levels besides to allow the selection of potential targets for the design of new therapeutic drugs. In this study, we employed a 2D-DIGE approach to identify proteins differentially abundant in phosphoprotein-enriched samples of *L. braziliensis*, seeking to analyze the impact of the phosphorylation on drug stress response and antimony resistance phenotype in this parasite. The 2D-DIGE is a powerful technique that allows simultaneous visualization of relatively large portions of the proteome, using mixtures of protein extracts labeled with different fluorescent CyDyes, which can be separated simultaneously on the same 2D gel [61]. Indeed, this technique enabled us the identification of 48 phosphoproteins, which were distributed into seven categories of biological process, with differential abundance among the samples analyzed (Figs. 2 and 3). Recently, comparative analysis of total proteome of

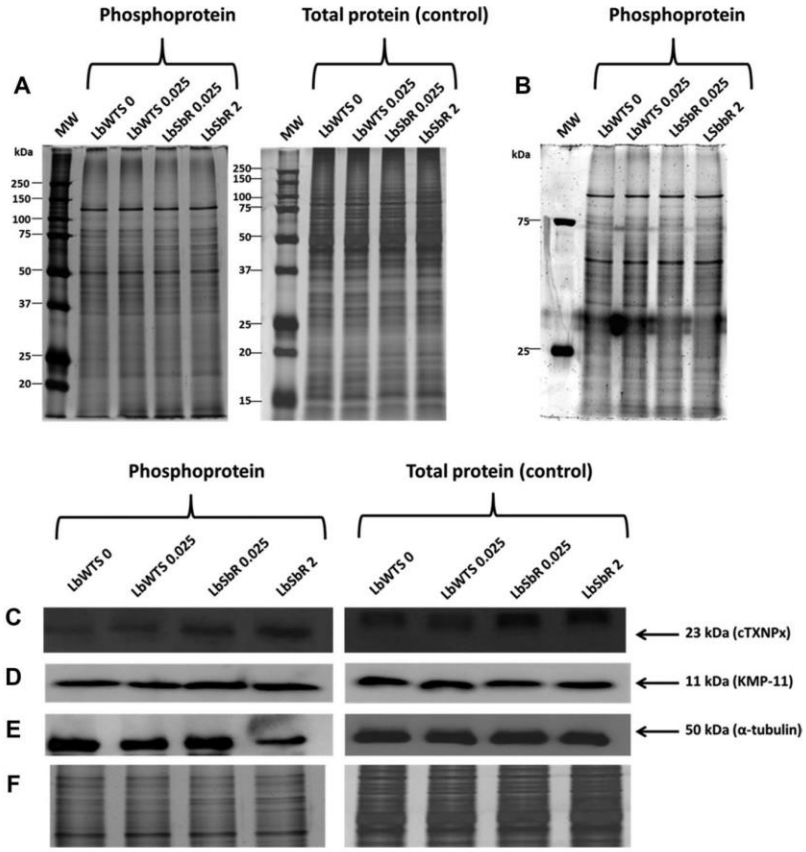

Figure 5. Analysis of the expression and phosphorylation levels of six phosphoproteins in *L. braziliensis* samples by Western blot assays. Total proteins (controls) and phosphoprotein extracts of the samples were separated by SDS-PAGE and stained with silver (A) and ProQ® Diamond Phosphoprotein Gel Stain (B). Western blot analysis—Samples (1 μg) were separated by SDS-PAGE, transferred to nitrocellulose membrane and subsequently revealed with *T. cruzi* anti-cytosolic tryparedoxin peroxidase (C), *L. braziliensis* anti-kinetoplastid membrane protein-11 (D) and anti-alpha-tubulin (E) antibodies. The bands shown were normalized using the gel stained with silver (F). Molecular weight (MW) markers are indicated. The results are representative of three independent experimental replicates.

SbIII-susceptible and -resistant *L. braziliensis* lines conducted by our group revealed that 32 distinct proteins were differentially modulated in these lines of the parasite [24]. Nine of these proteins were also found in this phosphoproteomic study, like HSP70, HSP83-1, alpha- and beta-tubulin, tryparedoxin peroxidase, PTR1, PCNA, rieske iron-sulfur protein, and paraflagellar rod protein 1D. These results demonstrate that these common proteins are modulated in the phosphoproteome as well as in the total proteome of our lines of *L. braziliensis*.

The changes in the phosphorylation of proteins in several different systems have been previously linked to stress response. Heat-shock proteins (HSPs) are among the most highly conserved proteins along the evolutionary tree [36]. These proteins are found in all living organisms, from bacteria to humans. HSPs have important functions in folding, secretion, assembly, intracellular localization, regulation, and degradation of other proteins [62]. Heat shock for example induces rapid dephosphorylation of ribosomal proteins in *Drosophila* [63], controls the eukaryotic initiation factors eIF-2α and eIF-4β by phosphorylation and dephosphorylation in HeLa cells, respectively [64], triggers rapid protein phosphorylation in soybean seedings [65], and induces increase in the level of protein tyrosine phosphorylation in cultured animal cells [66]. Nevertheless, the effect of drug exposure on protein phosphorylation has not been evaluated in the parasite *L. braziliensis*. Thus, the pair wise comparisons from groups 1 and 5 performed in this study showed that the phosphoproteins HSP70 and HSP83-1 were more abundant in LbWTS 0.025 and LbSbR 2 (Fig. 3A, Tables 2 and 6). This result suggests that the SbIII causes a general stress in the parasite, inducing the production of phosphorylated proteins involved in "protein folding/chaperones and stress response." The increased abundance in the phosphorylation levels of these HSPs (HSP70 and HSP83-1) could be due to elevated activity of protein kinases or decreased activity of phosphatases. Still, the higher expression of phosphatases can cause a reduction in the phosphorylation levels in order to regulate the ratio phosphorylated to unphosphorylated. Furthermore, according to Morales et al. [28] the absence of transcriptional regulation factors in trypanosomatids may have been compensated for by the evolution of protein kinases that regulate chaperone function, and thereby the major *Leishmania* chaperones may be promising drug targets by inhibition of their respective protein kinases. Moreover, several authors reported the overexpression of HSP70

and HSP83 in antimony-resistant isolates of *L. donovani* [23, 50, 51], *L. braziliensis*, and *L. infantum* lines [24, 44].

Interestingly, our phosphoproteomic data also indicated that several phosphoproteins were exclusively more abundant in the SbIII-resistant samples (LbSbR 0.025 and/or LbSbR 2) from the groups 2, 3, and 4, demonstrating that the regulation of these proteins by phosphorylation may be involved in important molecular process associated to the antimony resistance phenotype in *L. braziliensis* (Tables 3–5). The functional distribution of these phosphoproteins showed that they were implicated in all categories of biological process raised in this study (Fig. 3B). An important mechanism activated by components of oxidative phosphorylation pathway is the antioxidant defense. Indeed, we observed some phosphoproteins, such as tryparedoxin peroxidase, peroxidoxin, and pteridine reductase 1 with increased phosphorylation levels, contributing for reduction of the cell damage generated by drug stress. NDKb, a biosynthetic kinase, was found more abundant in the SbIII-resistant parasites, indicating that it may be associated to antimony resistance phenotype in *L. braziliensis*. We also observed increased abundance for certain phosphoproteins: proliferative cell nuclear antigen, Ran-binding protein 1, and nucleosome assembly protein, showing that the phosphorylation of these proteins could maintain uninterruptedly the DNA replication and repair in antimony-resistant samples. Other important mechanism that may also be activated by the phosphorylation is the category of "protein biosynthesis." This is corroborated by increase of translation initiation and ribosomal subunits, such as eukaryotic translation initiation factor 1A and 40S ribosomal protein S12. These phosphoproteins identified in our study suggest that they are involved in the production of essential proteins of SbIII-resistant *Leishmania*. Thus, the PTM of protein synthesis machinery by phosphorylation is important for its regulation and could be critical for survival of antimony-resistant parasites encountering stress. In addition, a high number of hypothetical proteins were observed in our analysis. Among these, two phosphoproteins (LbrM.33.0650 and LbrM.34.4450) were more abundant in the SbIII-resistant samples from the groups 2, 3, and 4 (Tables 3–5), demonstrating that the phosphorylation may also be associated in their regulation. Together, all these changes highlighted until here indicate that *Leishmania* probably increases the abundance of enzymes involved in these diverse pathways for its protection and reduction of the effects generated by drug pressure. Indeed, it has been demonstrated that physiological cellular events such as parasite infectivity, xenobiotics conjugation, and extrusion, drug transport, cytoskeletal phosphorylation, intracellular metabolism, host-parasite interaction, incorporation of metabolites essential for the parasite survival, promastigote-amastigote differentiation, and parasite cell shape may occur along with *Leishmania* drug-resistance [67]. Therefore, an elaborate phosphorylation signaling system to respond to these changes may be a fundamental strategy adopted by this parasite.

Interestingly, we observed in our phosphoproteomic analysis that the two spots with the biggest fold difference are PTR1 (LbrM.23.0300) and ASS (LbrM.23.0290). We performed Southern blot analysis to investigate the *ptr1* gene amplification. The results showed that the *ptr1* gene is amplified in the SbIII-resistant line of *L. braziliensis* (data not shown). Recently, previous results of our research group demonstrated that the *MRPA* (multidrug resistance protein A) gene is amplified in this same antimony-resistant *L. braziliensis* line [68]. The *MRPA* gene is part of the H locus, a region that also contains the *ptr1* gene [69,70]. Thus, these results support the idea that the *ptr1* gene is coamplified with *MRPA* gene in our LbSbR line. Although we did not perform analysis to evaluate the *ASS* amplification, previous study demonstrated that a short region containing the *MRPA* and *ASS* genes was amplified in antimony-resistant *L. infantum* by microarrays analysis [71]. It is important to highlight that the MRPA protein was not identified in this phosphoproteomic analysis due to its poor solubility in 2D gels, requiring specific protocols to obtain fractions enriched of membrane proteins [50].

The identification and quantification of phosphorylation sites in trypanosomatids is increasing in an attempt to determine their function and involvement in signaling pathways. The first phosphoproteomic study of the bloodstream from *Trypanosoma brucei* revealed more than 1204 p-sites on 491 proteins, with phosphorylation predominantly on serine (S) (75%), threonine (T) (21.5%), and tyrosine (Y) (3.5%) residues [72]. The analysis of the first global phosphoproteomic study of *T. cruzi* epimastigotes showed 220 p-sites on 119 phosphoproteins, with phosphorylation distributed between S (65.5%), T (25.2%), and Y (3.5%) residues [73]. Phosphoproteomic results of *L. donovani* axenic amastigotes demonstrated 181 p-sites in 126 distinct proteins, with phosphorylation predominantly on S (86%), T (12%), and Y (2%) residues [29]. The comparison of the phosphoproteomes of *L. donovani* amastigotes and promastigotes revealed 1614 p-sites corresponding to 627 proteins, with S phosphorylation significantly enriched in amastigotes [30]. These studies demonstrate that about one-thirds of phosphorylation events seem to occur on T residues in *T. brucei* and *T. cruzi* [72, 73], while phosphorylation occurring on S residues is predominant in *Leishmania* spp., which is similar to the distribution observed in higher eukaryotes [74, 75]. In this study, the phosphoproteomic technique used was unable to assign any specific p-sites in our *L. braziliensis* samples. Our preliminary analysis of multiple sequence alignments for some phosphoproteins found here revealed that the phosphorylation is common on S residues (Fig. 4), besides a conservation of the p-sites among the different *Leishmania* species, which may play important roles in diverse regulatory pathways in these parasites. Further investigation of the p-sites in our *L. braziliensis* samples using an adequate methodology is essential to elucidate signaling events associated to drug stress response and antimony resistance phenotype.

Phosphoproteomics studies represent an important method to record the dynamics of phosphorylation levels in response to perturbation, which can contribute to clarify signaling pathways in trypanosomatids. This novel study allowed

us to profile the *L. braziliensis* phosphoproteome, identifying several potential candidates for biochemical or signaling networks associated with antimony resistance phenotype in this parasite. Thus, we believe that our phosphoproteomic analysis can bring new insight into posttranslational mechanisms of the *L. braziliensis* stress response to antiparasitic treatment and drug resistance, contributing to understand the *Leishmania* biology and to drive drug-discovery efforts against the leishmaniasis. Moreover, future functional analysis will be performed to decipher key molecular events in signaling cascades regulating the SbIII resistance mechanisms in our phosphoprotein samples of *L. braziliensis*.

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The authors have declared no conflict of interest.

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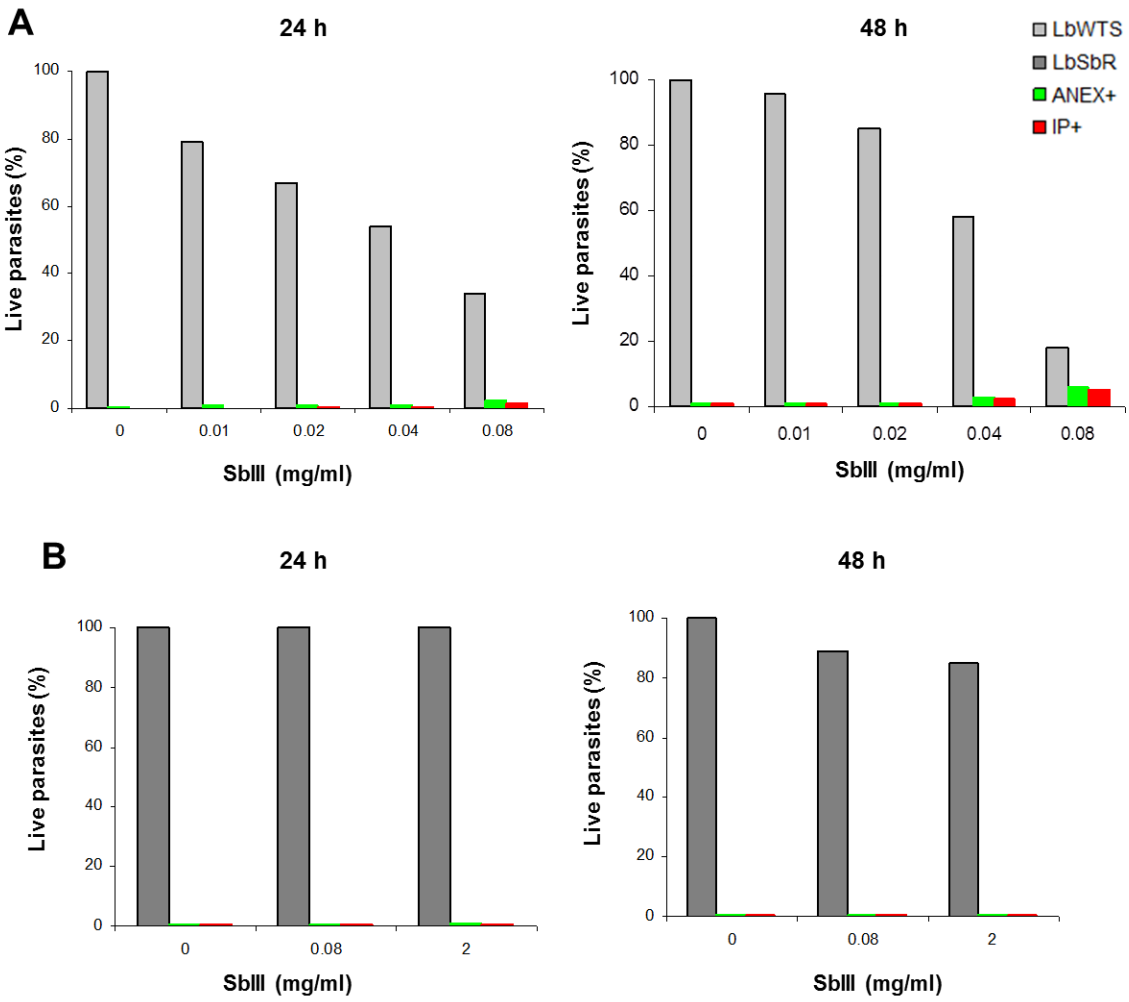
SUPPORTING INFORMATION

Phosphoproteomic analysis of wild-type and antimony-resistant *Leishmania braziliensis* lines by 2D-DIGE technology



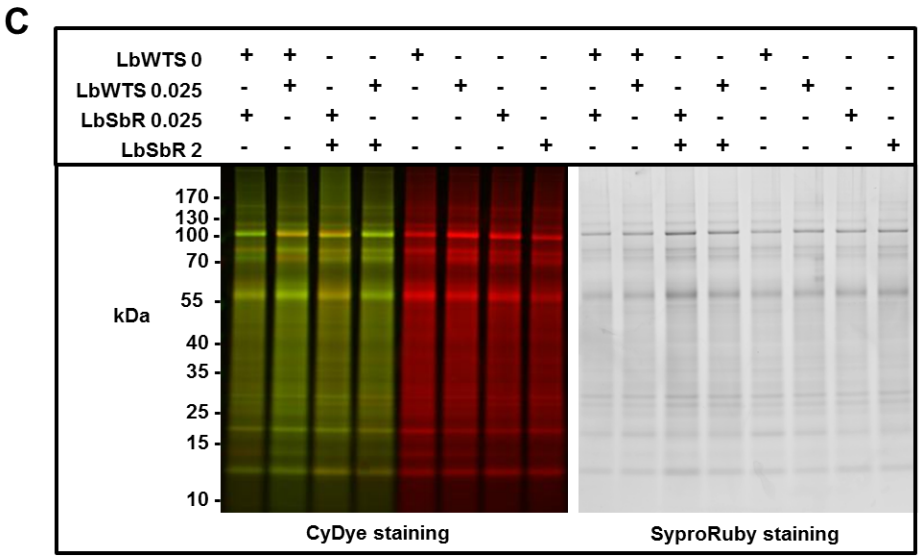


Fig. 1S. *Optimization and validation of experimental conditions.* Determination of parasite survival as a function of drug concentration by FACS analysis in Sb^{III}-susceptible (A) and -resistant (B) *L. braziliensis* lines. The parasites were cultivated by 24 and 48 hours in M199 medium containing different Sb^{III} concentrations (mg/ml). The parasite number was determined using a Z1 Coulter Counter (Beckman Coulter, Fullerton, CA, USA). After, 10^6 cells were incubated with annexin V (anex) during 10 min and propidium iodide (PI) by 5 min, according to protocol described by Annexin-V-FLUOS staining kit (Roche). Subsequently they were submitted to Flow cytometer analysis using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter > 600 nm for PI detection. Positive parasite number was represented in the plot. Two independent measurements, each performed in duplicate were performed. (C) 1D-DIGE analysis and SyproRuby staining. Phosphoprotein analysis of *L. braziliensis* samples Sb^{III} treated and non-treated. The red/orange bands are indicating differences in phosphoprotein abundance. This is a technical control of the protein amount that indeed is equal between samples. LbWTS 0 (Cy3) x LbSbR 0.025 (Cy5) – lane 1; LbWTS 0 (Cy5) x LbWTS 0.025 (Cy3) – lane 2; LbSbR 0.025 (Cy3) x LbSbR 2 (Cy5) – lane 3; LbWTS 0.025 (Cy5) x LbSbR 2 (Cy3) – lane 4; LbWTS 0 (Cy3) – lane 5; LbWTS 0.025 (Cy3) – lane 6; LbSbR 0.025 (Cy3) – lane 7; LbSbR 2 (Cy3) – lane 8.

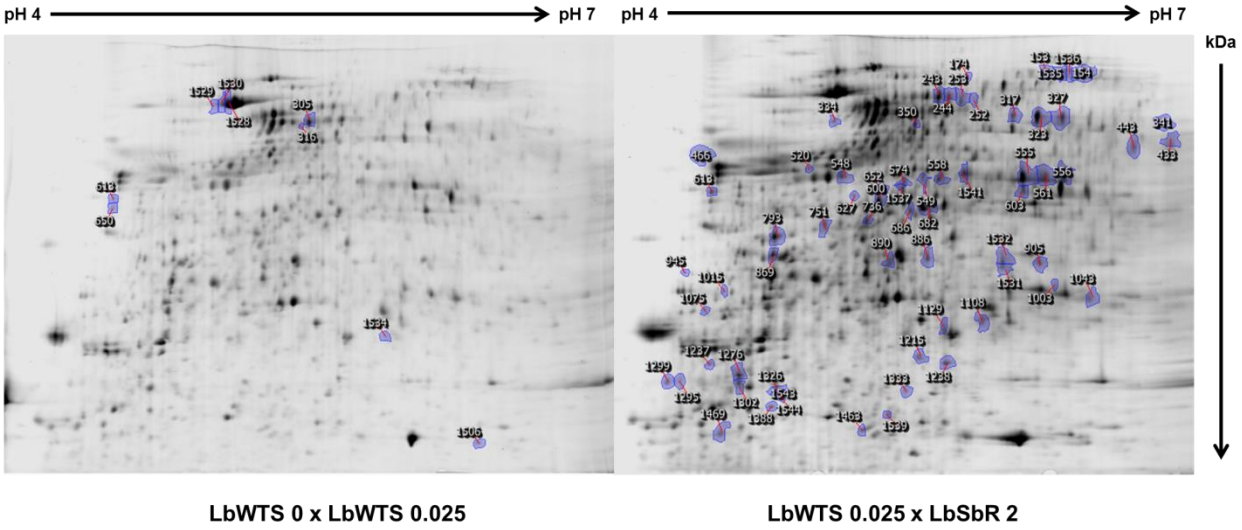


Fig. 2S. *Protein spots differentially abundant in the groups 1 (LbWTS 0 x LbWTS 0.025) and 4 (LbWTS 0.025 x LbSbR 2) of* L. braziliensis. Spots that showed a minimum of 1.5-fold change in fluorescence intensity with a *p*-value < 0.05 (ANOVA) were subjected to mass spectrometry (MS/MS). The numbers in the gels refer to the spot identification as shown in the Tables 2 and 5. Each gel is representative of four independent experimental replicates.

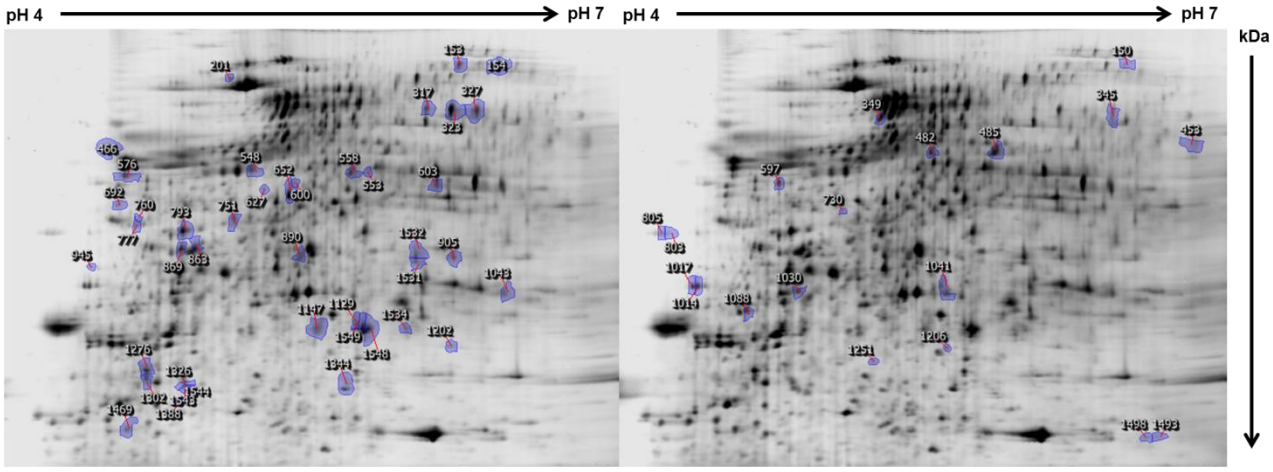


Fig. 3S. *Protein spots differentially abundant in the group 3 (LbWTS 0 x LbSbR 0.025) of* L. braziliensis. Spots that showed a minimum of 1.5-fold change in fluorescence intensity with a *p*-value < 0.05 (ANOVA) were subjected to mass spectrometry (MS/MS). The numbers in the gels refer to the spot identification as shown in the Table 4. Each gel is representative of four independent experimental replicates.

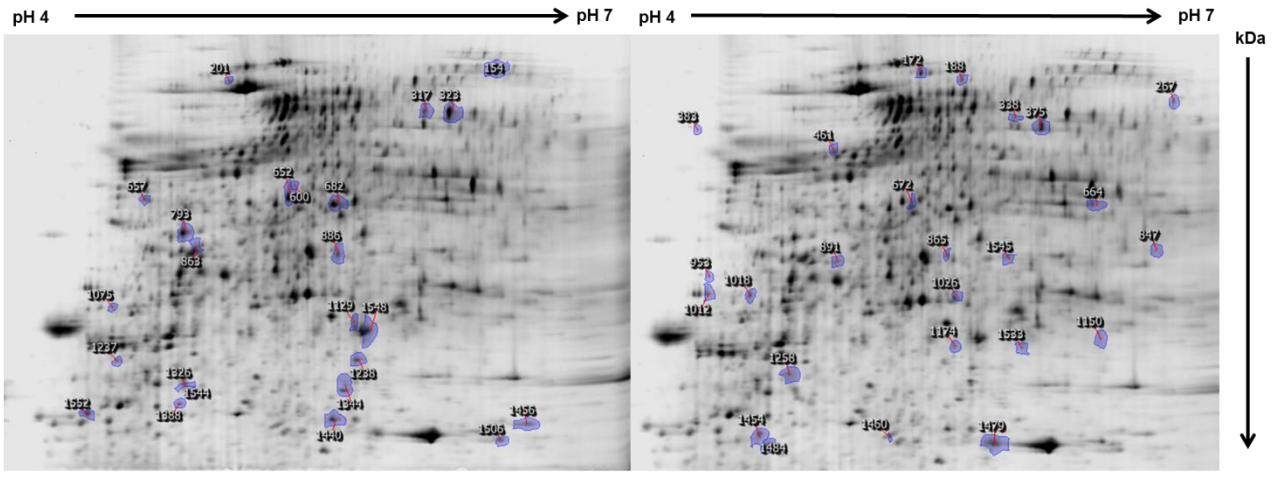
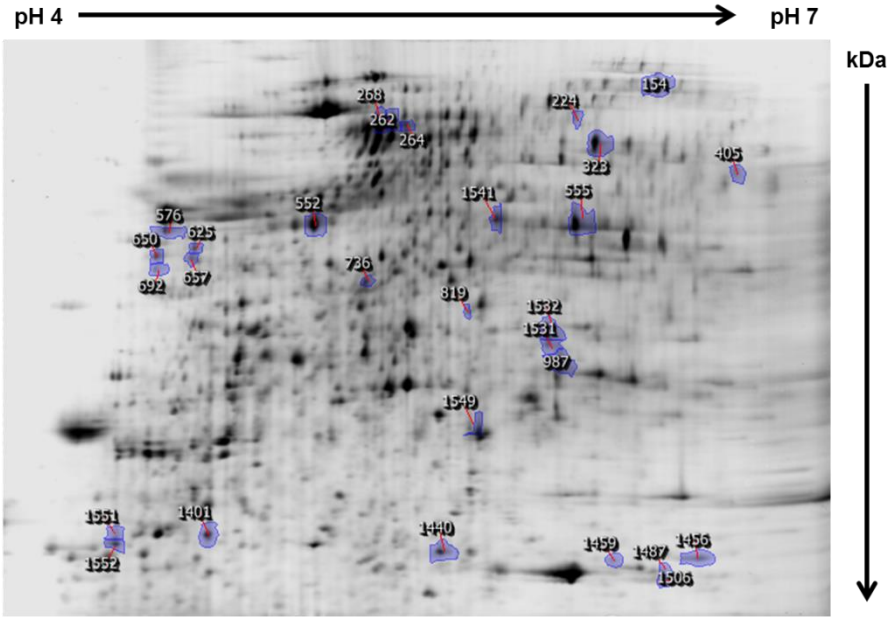


Fig. 4S. *Protein spots differentially abundant in the group 2 (LbWTS 0.025 x LbSbR 0.025) of* L. braziliensis. Spots that showed a minimum of 1.5-fold change in fluorescence intensity with a *p*-value < 0.05 (ANOVA) were subjected to mass spectrometry (MS/MS). The numbers in the gels refer to the spot identification as shown in the Table 3. Each gel is representative of four independent experimental replicates.



LbSbR 0.025 x LbSbR 2

Fig. 5S. *Protein spots differentially abundant in the group 5 (LbSbR 0.025 x LbSbR 2) of* L. braziliensis. Spots that showed a minimum of 1.5-fold change in fluorescence intensity with a *p*-value < 0.05 (ANOVA) were subjected to mass spectrometry (MS/MS). The numbers in the gels refer to the spot identification as shown in the Table 6. Each gel is representative of four independent experimental replicates.

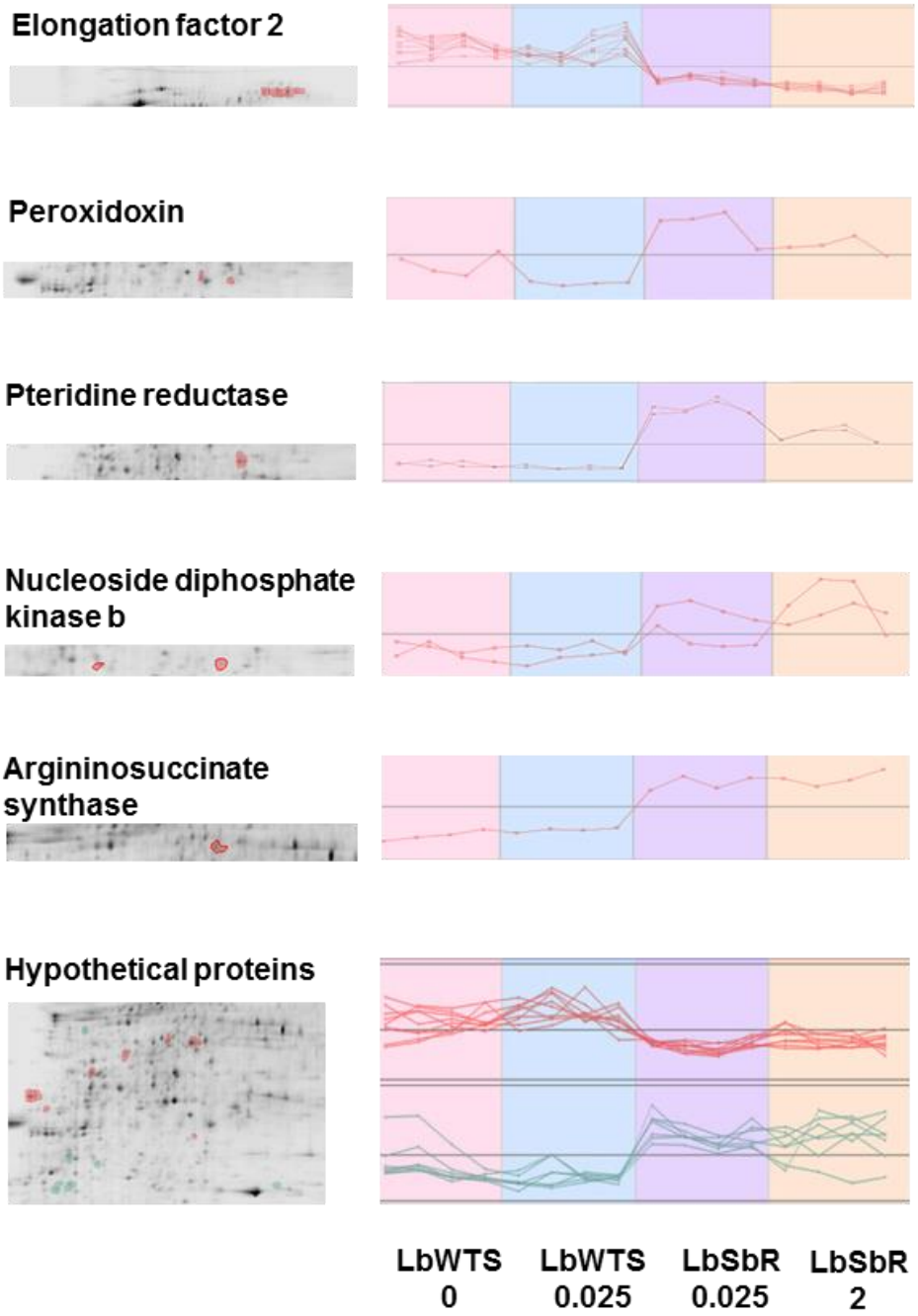


Fig. 6S. *Representative analysis of cross comparisons for some proteins*. The graphics reveal the abundance of proteins in our different *L. braziliensis* samples analyzed and informs on phosphoproteins that are linked to drug treatment or drug resistance. The analysis was performed using the Progenesis SameSpots software V4.1 (Nonlinear Dynamics, Newcastle upon Tyne, UK).

4.2 Artigo 2: Genômica funcional

Moreira and Murta *Parasites & Vectors* (2016) 9:641
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Parasites & Vectors

RESEARCH Open Access

Involvement of nucleoside diphosphate kinase b and elongation factor 2 in *Leishmania braziliensis* antimony resistance phenotype

Douglas S. Moreira and Silvane M. F. Murta*

Abstract

Background: Nucleoside diphosphate kinase b (NDKb) is responsible for nucleoside triphosphates synthesis and it has key role in the purine metabolism in trypanosomatid protozoans. Elongation factor 2 (EF2) is an important factor for protein synthesis. Recently, our phosphoproteomic analysis demonstrated that NDKb and EF2 proteins were phosphorylated and dephosphorylated in antimony (Sb^{III})-resistant *L. braziliensis* line compared to its Sb^{III}-susceptible pair, respectively.

Methods: In this study, the overexpression of *NDKb* and *EF2* genes in *L. braziliensis* and *L. infantum* was performed to investigate the contribution of these proteins in the Sb^{III}-resistance phenotype. Furthermore, we examined the role of lamivudine on Sb^{III} susceptibility in clones that overexpress the *NDKb* gene, and the effect of EF2 kinase (EF2K) inhibitor on the growth of EF2-overexpressing parasites.

Results: Western blot analysis demonstrated that NDKb and EF2 proteins are more and less expressed, respectively, in Sb^{III}-resistant line of *L. braziliensis* than its wild-type (WTS) counterpart, corroborating our previous phosphoproteomic data. NDKb or EF2-overexpressing *L. braziliensis* lines were 1.6 to 2.1-fold more resistant to Sb^{III} than the untransfected WTS line. In contrast, no difference in Sb^{III} susceptibility was observed in *L. infantum* parasites overexpressing NDKb or EF2. Susceptibility assays showed that NDKb-overexpressing *L. braziliensis* lines presented elevated resistance to lamivudine, an antiviral agent, but it did not alter the leishmanicidal activity in association with Sb^{III}. EF2-overexpressing *L. braziliensis* clone was slightly more resistant to EF2K inhibitor than the WTS line. Surprisingly, this inhibitor increased the antileishmanial effect of Sb^{III}, suggesting that this association might be a valuable strategy for leishmaniasis chemotherapy.

Conclusion: Our findings represent the first study of *NDKb* and *EF2* genes overexpression that demonstrates an increase of Sb^{III} resistance in *L. braziliensis* which can contribute to develop new strategies for leishmaniasis treatment.

Keywords: *Leishmania* spp., Nucleoside diphosphate kinase b, Elongation factor 2, Chemotherapy, Antimony resistance

* Correspondence: silvane@cpqrr.fiocruz.br
Centro de Pesquisas René Rachou CPqRR, Fundação Oswaldo Cruz - FIOCRUZ, Avenida Augusto de Lima 1715, Belo Horizonte, MG, Brazil

Background

Leishmaniasis refers to a disease complex caused by protozoan *Leishmania* parasites which are transmitted to humans by the bite of infected female phlebotomine sandflies. According to the World Health Organization (WHO), leishmaniasis is a neglected tropical disease that constitutes a public health problem in many developing countries of the Indian subcontinent, Latin America and East Africa [1]. Human leishmaniasis has an incidence of 1.2 million new cases annually, with an estimated population of 350 million at risk and a prevalence of 12 million cases [2]. Depending on genetic and environmental factors, the host immune response and mainly on *Leishmania* species involved, the disease can comprise three main clinical manifestations: cutaneous (CL), mucocutaneous (MCL) or visceral (VL) [3]. In the New World, *L.* (*Viannia*) *braziliensis* is the causative agent of CL and MCL, whereas *L.* (*Leishmania*) *infantum* [syn. *L.* (*L.*) *chagasi*] causes VL, which is lethal if not treated [4, 5].

Pentavalent antimonials (Sb^V), such as sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®), remain the first-line of treatment against all forms of the disease for more than 70 years especially in developing countries [6]. Despite the mechanism of antimony action has not been completely elucidated, studies suggest that Sb^V is reduced to trivalent form (Sb^{III}) that is active against amastigote and promastigote forms of *Leishmania* [7]. Earlier reports have indicated that antimonials inhibit fatty acid β-oxidation and glycolysis [8], and cause perturbations in the thiol redox potential, which would drive to parasite death by oxidative stress [9]. Furthermore, it has been suggested that antimony can kill the parasite by an apoptosis process resulting in DNA fragmentation and externalization of phosphatidylserine outside of *Leishmania* [10, 11].

The emergence and spread of resistance to antimony is significant in determined regions, such as Bihar state in India where over 60% of VL patients do not respond to the traditional therapy using antimonials [12]. Recent studies have demonstrated several mechanisms in *Leishmania* species implicated in resistance to these compounds. Thus, antimony resistance constitutes a multifactorial process and involves at least some of following aspects. A decrease in rate of reduction from pentavalent to trivalent form or loss of reductase activity may lead to drug resistance [13, 14]. Lower expression of AQP1 (aquaglyceroporin), which is involved in Sb^{III} uptake into parasite, was also observed in resistant mutants [15, 16]. Increased levels of intracellular thiols were found in cells selected for resistance to Sb^{III} [17] as well as in unresponsive clinical isolates [18–20]. MRPA (multidrug resistance associated protein A) transporter confers resistance by sequestering thiol-Sb conjugates into an intracellular vacuole which removes the drug from the cytoplasm of the parasite [21]. An increase of PGP (phosphoglycoprotein) expression was described in *Leishmania* resistant to antimonials [22], suggesting that this protein mediates the efflux of these drugs from the parasite. Moreover, other mechanisms can also contribute to the antimony resistance phenotype in *Leishmania*.

Nucleoside diphosphate kinase b (NDKb), a NDK family member, is ubiquitous enzyme that is crucial to transfer phosphate group from a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP), using a ping-pong mechanism that involves a phosphohistidine intermediate [23, 24]. The whole reaction is described as follows [25]:

$$\mathrm{NDK} + \mathrm{N_1TP} \leftrightarrow \mathrm{NDK\text{-}P} + \mathrm{N_1DP} \leftrightarrow \mathrm{NDK\text{-}P} + \mathrm{N_2DP} \leftrightarrow \mathrm{NDK} + \mathrm{N_2TP}$$

NDKs play pivotal roles in different organisms, such as in bacterial pathogenesis [26], regulation of gene expression in cells of mammals [27] and participation in the purine salvage pathways of protozoan parasites [28]. NTP is a precursor for DNA and RNA synthesis, evidencing that NDK is also an essential enzyme for all cellular processes involving nucleic acids in distinct species of organisms [24]. Unlike mammals, *Leishmania* is not able to synthesize purines by means of a *de novo* mechanism and thus depend upon the host for survival [29]. Kolli et al. [30] showed that *Leishmania*-released NDK avoids ATP-mediated lysis of macrophages, keeping the integrity of host cells to the advantage of the parasite. Therefore, NDKb can be considered an interesting target for drug discovery for chemotherapy of leishmaniasis.

The regulation of protein synthesis in eukaryotic cells occurs via both initiation and elongation levels. This process uses significant quantity of cellular energy, and the vast majority of this is consumed in elongation [31]. Elongation factor 2 (EF2) codifies a member of the GTP-binding translation elongation factor family and it is a relevant factor for production of proteins. EF2, which is a ubiquitous enzyme, makes the GTP-dependent translocation of the aminoacyl-tRNA from the A-site to the P-site of the ribosome [32]. EF2 protein was found with increased abundance in promastigote forms of *L. panamensis* resistant to Sb^{III} [33].

Considering the multiplicity of antimony resistance mechanisms, our knowledge about them in New World *Leishmania* species is far from being fully elucidated. Recently, our phosphoproteomic analysis demonstrated that the nucleoside diphosphate kinase b (NDKb) and elongation factor 2 (EF2) proteins were phosphorylated and dephosphorylated, respectively, in Sb^{III}-resistant *L. braziliensis* line compared to its wild-type (WTS) counterpart [34]. In this study, *NDKb* and *EF2* genes were transfected in Sb^{III}-susceptible lines of *L. braziliensis* and

L. infantum to determine whether the overexpression of these proteins contributes to antimony resistance phenotype in these parasites. Moreover, we investigated the role of lamivudine on Sb^{III} susceptibility in NDKb-overexpressing clones, and the effect of EF2 kinase (EF2K) inhibitor on the growth of EF2-overexpressing parasites.

Methods

Leishmania spp. cultures

We used promastigote forms of *L. braziliensis* (MHOM/BR/75/M2904) and *L. infantum* (MHOM/BR/74/PP75) in our study. The antimony-resistant lines were previously selected in vitro to potassium antimonyl tartrate (Sb^{III}) ($C_8H_4K_2O_{12}Sb_2.3H_2O$) by step-wise drug pressure and their resistance indices were 20-fold and 4-fold higher than those of their wild-type counterparts, respectively [35]. Parasites were grown at 26 °C in M199 medium supplemented with 2 mM L-glutamine, 5 μg/ml hemin, 50 μg/ml streptomycin, 2 μg/ml biopterin, 1 μg/ml biotin, 40 mM HEPES pH 7.4, 500 U penicillin and 10% v/v heat-inactivated fetal calf serum [35]. These parasites were harvested in the logarithmic growth phase to perform all analyses.

Generation of NDKb and EF2 overexpressing lines

A 456 bp fragment corresponding to *NDKb* encoding region (TriTrypDB accession number LbrM.32.3210) was amplified with *Pfx* DNA polymerase (Invitrogen) from *L. braziliensis* genomic DNA using the forward primer: 5′-TGG ATC CCC ACC ATG TCC TCC GAG CGC ACT TT-3′ and the reverse primer: 5′-TTG GAT CCC TAT TCG TAG ATC TGG CAA GCG G-3′. Other 2,538 bp fragment corresponding to *EF2* encoding region (TriTrypDB accession number LbrM.35.0270) was also amplified with the enzyme cited above using *L. braziliensis* genomic DNA and the primers forward: 5′-TGG ATC CCC ACC ATG GTG AAC TTT ACC GTC GAT CAG-3′ and reverse: 5′-TTG GAT CCT TAC AAT TTA TCC ATG AAC TGG TCC A-3′. The underlined sequences correspond to *Bam*HI restriction site. The obtained PCR products were cloned into the pGEM-T Easy® vector (Promega, Madison, WI, USA) and subsequently submitted to sequencing reaction for confirmation of correct sequence. All constructs were sequenced in an ABI 3130 (Applied Biosystems, Foster City, CA, USA). The pGEM-NDKb and pGEM-EF2 constructs were restricted with *Bam*HI and the fragments released were subcloned into the dephosphorylated pIR1BSD expression vector (kindly provided by Dr. Stephen Beverley, Washington University, USA). To confirm the correct direction of cloning, the constructs were then digested with *Hind*III and *Sma*I releasing fragments that confirmed the sense direction of the genes *NDKb* and *EF2*, respectively. Thus, the constructs pIR1BSD (empty vector), pIR1BSD-NDKb and pIR1BSD-EF2 were linearized by *Swa*I digestion and electroporated into wild-type *L. braziliensis* and *L. infantum* lines using a GenePulser XCell electroporation system (Bio-Rad, Hercules, CA, USA). This allowed integration of the vector into the 18S ribosomal DNA small subunit locus [36]. Colonies were obtained following plating on semisolid M199 medium containing blasticidin (BSD) (10 μg/ml). After 1–2 weeks, clonal lines were selected and the presence of constructs was confirmed by PCR tests using genomic DNA with primers specific for the BSD marker.

Protein levels

Western blot assays were carried out for investigating the expression level of NDKb and EF2 proteins in the transfected parasites and in the Sb^{III}-resistant and -susceptible lines of *L. braziliensis* and *L. infantum*. Total proteins from these parasites were extracted according to the protocol previously described [37]. Subsequently, 20 μg from each sample were separated by electrophoresis on 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). They were blocked, washed and probed with rabbit polyclonal anti-NDKb (1:200) (Abcam, Cambridge, UK, #ab154274) or rabbit monoclonal anti-EF2 (1:200) (Abcam, Cambridge, UK, #ab75748) antibodies, during 12 h at 4 °C in the blocking solution. According to manufacturer specifications, the immunogen of the first antibody is a recombinant fragment corresponding to a region within amino acids 1–105 of human NDKb, while the immunogen of the second antibody is a synthetic peptide corresponding to residues on the C terminal of human EF2 (Abcam, Cambridge, UK). The blots were washed twice and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000) (GE Healthcare) for 1 h at room temperature. After incubation, the membranes were washed, incubated with ECL Plus chemiluminescent substrate (GE Healthcare) and revealed by ImageQuant LAS 4000 (GE Healthcare). To confirm equivalent loading, SDS-PAGE containing the samples were stained with Coomassie blue. Furthermore, the blots were normalized using the antibody monoclonal anti-α-tubulin (1:15,000) (Sigma, St. Louis, USA). The intensity of the bands was analyzed using the software GelAnalyzer 2010 (gelanalyzer.com).

Susceptibility assays of *Leishmania* spp. clonal lines to Sb^{III} and H_2O_2

Promastigotes of wild-type *L. braziliensis* and *L. infantum* clonal lines non-transfected or transfected with the constructs pIR1BSD (empty vector), pIR1BSD-NDKb or pIR1BSD-EF2 were submitted to Sb^{III} (Sigma-Aldrich,

St. Louis, MO, USA) susceptibility tests. The susceptibility to hydrogen peroxide (H_2O_2) was also evaluated in the parasites transfected with the *NDKb* gene. Parasites were incubated in M199 medium at 2×10^6 cells/ml into 24-well plates in the absence or presence of several concentrations of Sb^{III} (1.17 to 599.04 μM which corresponds to 0.00078125 to 0.4 mg/ml) or H_2O_2 (100 to 350 μM) for 48 h. The effective concentration required to decrease growth by 50% (EC_{50}) was determined using a model Z1 Coulter Counter (Beckman Coulter, Fullerton, CA, USA). EC_{50} values were determined from at least three independent measurements performed in triplicate, using the linear interpolation method [38].

Susceptibility assays of *L. braziliensis* lines to lamivudine and EF2K inhibitor and competition assays

We determined the susceptibility of *L. braziliensis* lines to lamivudine (2′,3′-dideoxy-3′-thiacytidine - $C_8H_{11}N_3O_3S$) (Globe Química) (kindly provided by Dr. Juliana Medeiros and Dr. Ana Cláudia Tavares, Farmanguinhos/FIOCRUZ, Brazil) and 7-Amino-1-cyclopropyl-3-ethyl-1,2,3,4-tetrahydro-2,4-dioxopyrido [2,3-*d*] pyrimidine-6-carboxamide ($C_{13}H_{15}N_5O_3$) (Tocris Bioscience, A484954) which are inhibitors for the enzymes NDKb and elongation factor 2 kinase (EF2K), respectively. EC_{50} of these inhibitors for wild-type *L. braziliensis* line and parasites that overexpress the *NDKb* or *EF2* genes was determined to be used in competition tests with Sb^{III}. The cultures (2×10^6 cells/ml) were incubated in the absence or presence of various concentrations of lamivudine (125 to 10,000 μM) or EF2K inhibitor (50 to 300 μM) into 24-well plates during 48 h. After, the percentage of relative growth was determined by automated cell counting. Competition assays were performed to investigate the leishmanicidal activity of the inhibitors cited above in association with Sb^{III}. In these experiments, 2×10^6 parasites/ml were seeded into 24-well cell culture plates containing medium M199. Subsequently, the EC_{50} of lamivudine and EF2K inhibitor were added concomitantly with Sb^{III} EC_{50} (Table 1), followed by incubation for 48 h. The percentage of relative growth was determined by automated cell counting using a Z1 Coulter Counter.

Statistical analysis

Data were analyzed by Student's *t*-test performed using the software GraphPad Prism 5.0. A *P*-value less than 0.05 was considered statistically significant.

Results

Expression levels of NDKb and EF2 proteins in *Leishmania* lines

We determined the expression levels of the proteins NDKb and EF2 in the antimony-susceptible and antimony-resistant *L. braziliensis* and *L. infantum* lines by Western blot analysis using polyclonal anti-NDKb and monoclonal anti-EF2 antibodies, respectively. These antibodies are specific for mammalian proteins. It is important to highlight that the identity between the NDKb and EF2 amino acid sequences of *L. braziliensis* compared to mammalian was 66 and 61%, respectively (data not shown). Western blot results revealed that these antibodies recognized polypeptides of 17 kDa and 94 kDa in all *Leishmania* samples analyzed which correspond to the expected size of NDKb and EF2 proteins, respectively (Fig. 1a). The membranes were incubated with the monoclonal anti-α-tubulin antibody for normalization of the results (Fig. 1a). The expression level of NDKb protein was 1.5-fold and 2.4-fold higher in the Sb^{III}-resistant *Leishmania* spp. lines (LbSbR and LiSbR) in comparison with their respective wild-type lines (LbWTS and LiWTS) (Fig. 1a). Regarding expression level of EF2 protein, the results demonstrated that this protein was approximately 3-fold lower in the Sb^{III}-resistant *L. braziliensis* line when compared to its wild-type counterpart LbWTS. On the other hand, the EF2

Table 1 EC_{50} of Sb^{III}, lamivudine, EF2K inhibitor and hydrogen peroxide and corresponding RI for WTS, NDKb- or EF2-overexpressing *L. braziliensis* lines. EC_{50} represents effective concentration required to decrease growth by 50%. The values were determined from at least three independent experiments performed in triplicate, using the linear interpolation method [38]

Parasites	Sb^{III}		Lamivudine		EF2K inhibitor		Hydrogen peroxide	
	EC_{50} (μM)	RI	EC_{50} (μM)	RI	EC_{50} (μM)	RI	EC_{50} (μM)	RI
LbWTS	7	–	766	–	173	–	213	–
LbpIR1BSD	8	–	733	–	–	–	207	–
LbNDKb clone 4	12	1.7	2,110	2.8	–	–	207	1.0
LbNDKb clone 9	15	2.1	2,014	2.6	–	–	207	1.0
LbEF2 clone 9	14	2.0	–	–	211	1.2	–	–
LbEF2 clone 12	11	1.6	–	–	–	–	–	–

Chemical formulae and abbreviations: Sb^{III} (potassium antimonyl tartrate); lamivudine ($C_8H_{11}N_3O_3S$); EF2K inhibitor ($C_{13}H_{15}N_5O_3$); hydrogen peroxide (H_2O_2); *WTS* wild-type susceptible, *Lb L.* (*V.*) *braziliensis*, *pIR1BSD* expression vector, *NDKb* nucleoside diphosphate kinase b, *EF2* elongation factor 2, *EF2K* elongation factor 2 kinase, *RI* resistance index

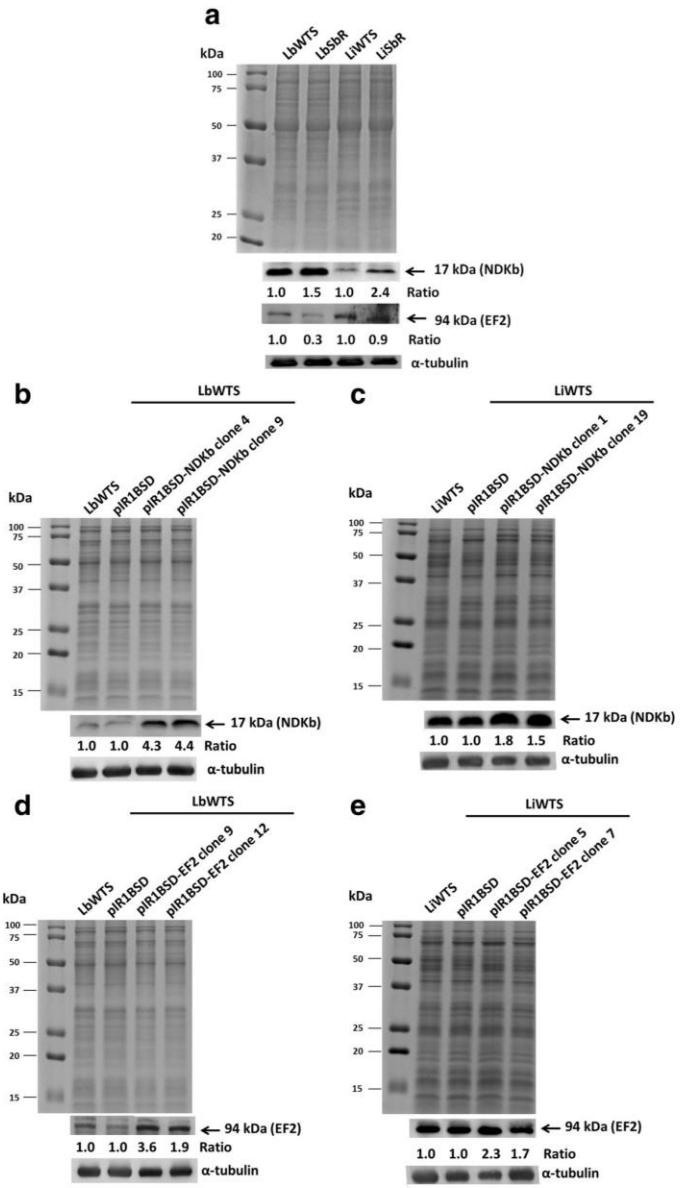

Fig. 1 NDKb and EF2 expression levels in wild-type (WTS), Sb^{III}-resistant (SbR) and clonal lines from *L. braziliensis* and *L. infantum* untransfected or transfected with the constructs pIR1BSD (empty vector), pIR1BSD-NDKb or pIR1BSD-EF2. Total proteins (20 μg) were separated by electrophoresis on 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The profiles of total proteins stained with Coomassie blue are shown. The blots were probed with rabbit polyclonal anti-NDKb (1:200) (**a**, **b** and **c**) or rabbit monoclonal anti-EF2 (1:200) (**a**, **d** and **e**) antibodies and developed using ECL Plus kit. All membranes were incubated with the anti-α-tubulin monoclonal (1:15,000) antibody for normalization of the results. Quantification of the bands was done by densitometric analysis using the software GelAnalyzer 2010. The figure is representative of all results obtained from two different biological replicates of each sample. The values of each replicate were used to calculate the averages and for determination of the ratios presented for each protein analyzed

protein presented the same level of expression between the LiWTS and LiSbR lines (Fig. 1a).

Overexpression of *NDKb* and *EF2* genes in *L. braziliensis* and *L. infantum*

Wild-type *L. braziliensis* and *L. infantum* lines were transfected with the constructs containing the *NDKb* or *EF2* genes (pIR1BSD-NDKb or pIR1BSD-EF2) and empty vector (pIR1BSD) to generate parasites overexpressing the enzymes NDKb and EF2. Linearization of this vector allowed integration of the constructs into the ribosomal small subunit locus [36]. To confirm the transfection, genomic DNA from the transfected clones was subjected to PCR assays using specific primers for the *BSD* gene, which confers resistance to blasticidin. It was observed that all blasticidin-resistant clones showed a fragment of 399 bp, which corresponds to BSD marker (data not shown). These clones were subjected to Western blot assays in order to evaluate if the NDKb and EF2 enzymes were overexpressed. Our results showed that the level of NDKb protein expression was 1.5 to 4.4-fold higher in the transfected clones from *L. braziliensis* and *L. infantum* lines than in the non-transfected or transfected with empty vector (controls) (Fig. 1b, c). Furthermore, our analysis also demonstrated that the expression level of EF2 protein was increased 1.7 to 3.6-fold in the transfected clones from these both wild-type *Leishmania* lines when compared to their respective controls (Fig. 1d, e).

Susceptibility of NDKb and EF2 overexpressing *Leishmania* spp. lines to Sb^{III}

We also investigated whether the overexpression of *NDKb* and *EF2* genes contributes to antimony resistance phenotype in *Leishmania*. For this, clonal lines from *L. braziliensis* and *L. infantum* transfected with the constructs pIR1BSD (empty vector), pIR1BSD-NDKb or pIR1BSD-EF2 and untransfected parasites were incubated with different Sb^{III} concentrations. The EC_{50} was determined by counting of parasites number grown in the absence or presence of this drug. The results showed that the Sb^{III} EC_{50} of untransfected *L. braziliensis* line was 7 μM. On the other hand, the clones 4 and 9 that overexpress NDKb in this *Leishmania* species presented EC_{50} of 12 μM and 15 μM, demonstrating an increase of 1.7 and 2.1-fold in the Sb^{III} resistance index of these clones, respectively (Fig. 2a) (Table 1). In contrast, NDKb-overexpressing *L. infantum* lines did not show an increase in resistance towards Sb^{III}. The results revealed that the Sb^{III} EC_{50} of wild-type *L. infantum* line was 67 μM, and the NDKb overexpressing clones 1 and 19 was 81 and 73 μM, respectively (Fig. 2b).

Regarding *EF2* gene, we observed that Sb^{III} EC_{50} of EF2-overexpressing clones 9 and 12 in *L. braziliensis* was 14 μM and 11 μM, showing an increase of 2.0 and 1.6-fold in the resistance index of Sb^{III} of these clones, respectively (Fig. 3a) (Table 1). On the other hand, EF2-overexpressing *L. infantum* lines did not alter the Sb^{III} susceptibility. The Sb^{III} EC_{50} of EF2-overexpressing clones 5 and 7 was 80 μM and 67 μM, respectively (Fig. 3b).

It is important to mention that NDKb- or EF2-overexpressing parasites presented similar growth curves when compared with their respective controls for both *Leishmania* species (data not shown). In the Sb^{III} susceptibility assays, we observed gradual reduction in the growth of NDKb- or EF2-overexpressing parasites of *L. braziliensis* with increasing drug concentrations. In

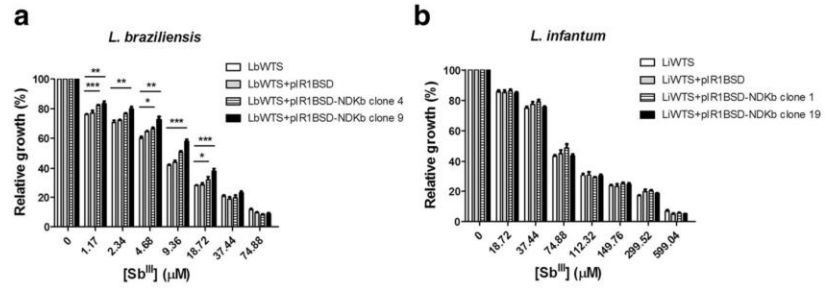

Fig. 2 Sb^{III} susceptibility assay of clonal lines from *L. braziliensis* (**a**) and *L. infantum* (**b**) non-transfected or transfected with the constructs pIR1BSD or pIR1BSD-NDKb. Parasites were incubated in M199 medium in the absence or presence of different concentrations of Sb^{III} (1.17 to 599.04 μM) for 48 h and the percentage of relative growth was determined using a Z1 Coulter Counter. Mean values ± standard deviations from three independent experiments in triplicate are shown. Statistical analysis was carried out using Student's *t*-test. Statistically different values are highlighted as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Pairwise comparisons (**a**): **1.17 μM:** LbWTS *vs* LbNDKb clone 4 ($t_{(5)} = 9.47$, $P = 0.0002$); LbWTS *vs* LbNDKb clone 9 ($t_{(9)} = 3.36$, $P = 0.0084$); **2.34 μM**: LbWTS *vs* LbNDKb clone 4 ($t_{(5)} = 4.3$, $P = 0.0077$); LbWTS *vs* LbNDKb clone 9 ($t_{(8)} = 3.74$, $P = 0.0057$); **4.68 μM:** LbWTS *vs* LbNDKb clone 4 ($t_{(4)} = 4.02$, $P = 0.0159$); LbWTS *vs* LbNDKb clone 9 ($t_{(5)} = 4.99$, $P = 0.0042$); **9.36 μM:** LbWTS *vs* LbNDKb clone 4 ($t_{(6)} = 8.39$, $P = 0.0002$); LbWTS *vs* LbNDKb clone 9 ($t_{(8)} = 12.50$, $P < 0.0001$); **18.72 μM:** LbWTS *vs* LbNDKb clone 4 ($t_{(8)} = 2.88$, $P = 0.0206$); LbWTS *vs* LbNDKb clone 9 ($t_{(10)} = 7.36$, $P < 0.0001$)

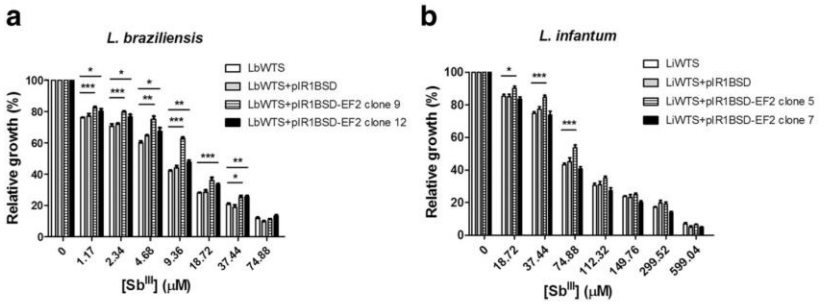

Fig. 3 Sb^{III} susceptibility assay of clonal lines from *L. braziliensis* (**a**) and *L. infantum* (**b**) non-transfected or transfected with the constructs pIR1BSD or pIR1BSD-EF2. Parasites were incubated in M199 medium in the absence or presence of different concentrations of Sb^{III} (1.17 to 599.04 μM) for 48 h and the percentage of relative growth was determined using a Z1 Coulter Counter. Mean values ± standard deviations from three independent experiments in triplicate are shown. Statistical analysis was carried out using Student's *t*-test. Statistically different values are highlighted as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Pairwise comparisons (**a**): **1.17 μM:** LbWTS *vs* LbEF2 clone 9 ($t_{(9)} = 6.5$, $P = 0.0001$); LbWTS *vs* LbEF2 clone 12 ($t_{(5)} = 2.84$, $P = 0.0361$); **2.34 μM**: LbWTS *vs* LbEF2 clone 9 ($t_{(7)} = 6.95$, $P = 0.0002$); LbWTS *vs* LbEF2 clone 12 ($t_{(4)} = 2.87$, $P = 0.0455$); **4.68 μM:** LbWTS *vs* LbEF2 clone 9 ($t_{(5)} = 6.04$, $P = 0.0018$); LbWTS *vs* LbEF2 clone 12 ($t_{(4)} = 2.87$, $P = 0.0453$); **9.36 μM:** LbWTS *vs* LbEF2 clone 9 ($t_{(7)} = 22.18$, $P < 0.0001$); LbWTS *vs* LbEF2 clone 12 ($t_{(8)} = 4.48$, $P = 0.0021$); **18.72 μM:** LbWTS *vs* LbEF2 clone 9 ($t_{(11)} = 4.74$, $P = 0.0006$); LbWTS vs. LbEF2 clone 12 ($t_{(9)} = 7.65$, $P < 0.0001$); **37.44 μM:** LbWTS *vs* LbEF2 clone 9 ($t_{(5)} = 2.78$, $P = 0.0391$); LbWTS *vs* LbEF2 clone 12 ($t_{(5)} = 5.1$, $P = 0.0038$). (**b**) **18.72 μM:** LiWTS *vs* LiEF2 clone 5 ($t_{(9)} = 2.67$, $P = 0.0257$); **37.44 μM:** LiWTS *vs* LiEF2 clone 5 ($t_{(8)} = 6.14$, $P = 0.0003$); **74.88 μM:** LiWTS *vs* LiEF2 clone 5 ($t_{(9)} = 4.9$, $P = 0.0008$)

addition, we observed that the overexpressors of NDKb or EF2 were more resistant to Sb^{III} until concentrations near to EC_{50} of these clones. In higher antimony concentrations, these parasites presented similar growth to their controls. We also observed that the vector used in our assays did not interfere in the Sb^{III} susceptibility since no difference in Sb^{III} EC_{50} was observed between parasites untransfected and transfected with empty vector.

Effect of lamivudine on the growth of wild-type and NDKb-overexpressing *L. braziliensis* lines

Initially, the amino acid sequence of NDKb (TriTrypDB accession number LbrM.32.3210) was used to search possible drugs against this enzyme in the DrugBank (www.drugbank.ca). Search results for the submitted sequence returned three antiviral agents: tenofovir (ID DB00300), lamivudine (ID DB00709) and adefovir dipivoxil (ID DB00718). These drugs are recommended in the chemotherapy of chronic hepatitis B (HBV), and the first two are also useful to treat HIV infection. Lamivudine is a synthetic nucleoside analogue and is phosphorylated intracellularly to its active 5′-triphosphate metabolite (lamivudine triphosphate - LTP), which is included into viral DNA by HIV reverse transcriptase and HBV polymerase, causing the ending of DNA chain (www.drugbank.ca/drugs/DB00709). We determined the lamivudine EC_{50} of wild-type *L. braziliensis* line and parasites transfected with the constructs pIR1BSD (empty vector) and pIR1BSD-NDKb. Our data showed that the lamivudine EC_{50} were 2.6-fold and 2.8-fold higher for NDKb-overexpressing clones 9 and 4 (2,014 and 2,110 μM, respectively) when compared to LbWTS (766 μM) (Fig. 4a) (Table 1), demonstrating that these clones are more resistant to lamivudine. Competition assay was carried out to analyze the combination of lamivudine and Sb^{III} treatment in WTS and NDKb-overexpressing *L. braziliensis* lines. Then, the parasites were incubated simultaneously with their respective EC_{50} for each drug (Table 1), and after 48 h the relative growth of the parasites was calculated. The combined effect of both compounds reduced by 55%, 59.7% and 63.7%, respectively, parasite numbers of LbWTS, LbNDKb clones 4 and 9, showing that the lamivudine did not increase the leishmanicidal activity of Sb^{III} (Fig. 4b).

Effect of EF2K inhibitor on the growth of wild-type and EF2-overexpressing *L. braziliensis* lines

Elongation factor 2 kinase (EF2K), a calmodulin-dependent protein, binds numerous up-stream signals to the regulation of protein synthesis. Thus, EF2K phosphorylates EF2 and inhibits the function of this enzyme [39]. Chen et al. [40] showed that inhibition of EF2K by EF2K inhibitor decreases EF2 phosphorylation however it has little effect on proliferation in the cancer cells. Then, we tested the potential leishmanicidal effect of this inhibitor against wild-type and EF2-overexpressing *L. braziliensis* lines. Incubation of parasites with different concentrations of EF2K inhibitor revealed that the EC_{50} of this drug was 1.2-fold higher for LbEF2 clone 9 (211 μM) in comparison with LbWTS (173 μM) (Fig. 5a) (Table 1). This result shows that the overexpression of EF2 enzyme protects

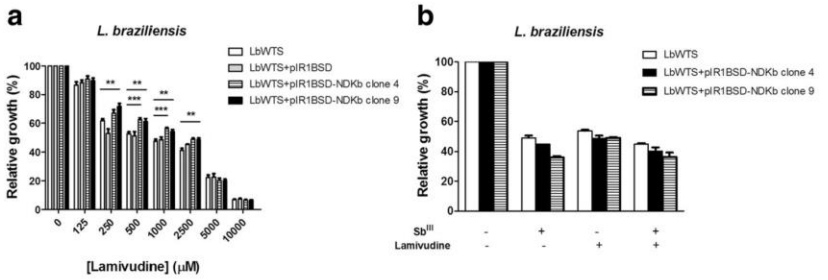

Fig. 4 EC_{50} of lamivudine for wild-type and NDKb-overexpressing *L. braziliensis* lines (**a**) and effect of lamivudine on the growth of *L. braziliensis* lines upon Sb^{III} exposure (**b**). Parasites were incubated in M199 medium in the absence or presence of different concentrations of lamivudine ($C_8H_{11}N_3O_3S$) (125 to 10,000 μM). For competition assay, cells were exposed to the EC_{50} of Sb^{III} (7, 12 and 15 μM for the LbWTS and NDKb-overexpressing clones 4 and 9, respectively) and the EC_{50} of lamivudine (766, 2110 and 2014 μM for the LbWTS and NDKb-overexpressing clones 4 and 9, respectively) independently or combined, followed by incubation for 48 h. The percentage of relative growth was determined using a Z1 Coulter Counter. Mean values ± standard deviations from three independent experiments in triplicate are shown. Statistical analysis was carried out using Student's *t*-test. Statistically different values are highlighted as follows: $^{**}P < 0.01$; $^{***}P < 0.001$. Pairwise comparisons (**a**): **250 μM:** LbWTS *vs* LbNDKb clone 9 ($t_{(7)} = 4.04$, $P = 0.0049$); **500 μM:** LbWTS *vs* LbNDKb clone 4 ($t_{(9)} = 5.27$, $P = 0.0005$); LbWTS *vs* LbNDKb clone 9 ($t_{(9)} = 3.74$, $P = 0.0046$); **1000 μM:** LbWTS *vs* LbNDKb clone 4 ($t_{(10)} = 5.67$, $P = 0.0002$); LbWTS *vs* LbNDKb clone 9 ($t_{(10)} = 3.52$, $P = 0.0055$); **2500 μM:** LbWTS *vs* LbNDKb clone 4 ($t_{(8)} = 5.03$, $P = 0.0010$); LbWTS *vs* LbNDKb clone 9 ($t_{(8)} = 3.91$, $P = 0.0045$)

slightly *Leishmania* from lethal action of EF2K inhibitor. In addition, we evaluated the effect of EF2K inhibitor on the growth of *L. braziliensis* lines upon Sb^{III} exposure. Surprisingly, the combined treatment Sb^{III} with EF2K inhibitor enhanced the leishmanicidal activity against both *L. braziliensis* lines compared to those incubated with Sb^{III} or EF2K inhibitor alone (Fig. 5b). Additionally, this increased Sb^{III} susceptibility was higher in the EF2-overexpressing *L. braziliensis* clone 9 (88% growth inhibition) than in the wild-type *L. braziliensis* line (77.7% growth inhibition), suggesting a possible involvement of EF2 in this activity.

Susceptibility of NDKb-overexpressing *L. braziliensis* lines to H_2O_2

L. braziliensis clonal lines overexpressing *NDKb* gene were submitted to susceptibility assays with H_2O_2 to evaluate the tolerance to oxidative stress generated by different concentrations of this compound. Our results demonstrated that LbWTS line showed an H_2O_2 EC_{50} of 213 μM, and the LbNDKb clones 4 and 9 displayed the same value of EC_{50} for H_2O_2, which was equal to 207 μM (Table 1). These data suggest that NDKb enzyme is not directly involved in the defense against oxidative stress in *L. braziliensis*.

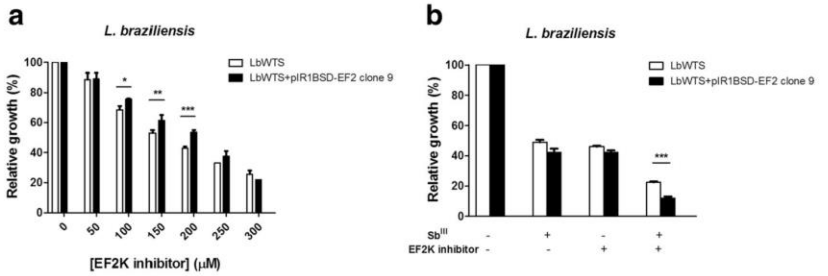

Fig. 5 EC_{50} of elongation factor 2 kinase (EF2K) inhibitor for wild-type and EF2-overexpressing *L. braziliensis* lines (**a**) and effect of EF2K inhibitor on the growth of *L. braziliensis* lines upon Sb^{III} exposure (**b**). Parasites were incubated in M199 medium in the absence or presence of different concentrations of EF2K inhibitor ($C_{13}H_{15}N_5O_3$) (50 to 300 μM). For competition assay, cells were exposed to the EC_{50} of Sb^{III} (7 and 14 μM for the LbWTS and EF2-overexpressing clone 9, respectively) and the EC_{50} of EF2K inhibitor (173 and 211 μM for the LbWTS and EF2-overexpressing clone 9, respectively) independently or combined, followed by incubation for 48 h. The percentage of relative growth was determined using a Z1 Coulter Counter. Mean values ± standard deviations from three independent experiments in triplicate are shown. Statistical analysis was carried out using Student's *t*-test. Statistically different values are highlighted as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Pairwise comparisons (**a**): **100 μM:** $t_{(6)} = 3.52$, $P = 0.0126$; **150 μM:** $t_{(6)} = 3.94$, $P = 0.0076$; **200 μM:** $t_{(6)} = 8.83$, $P = 0.0001$. (**b**) **Sb^{III} + EF2K inhibitor:** $t_{(4)} = 11.72$, $P = 0.0003$

Discussion

In the absence of effective vaccine against leishmaniasis, the only way to treat and control all forms of the disease is through the use of chemotherapy. Pentavalent antimonials are considered one of the main options of treatment; however these drugs have several toxic side effects and high resistance rates. Drug resistance in leishmaniasis has a multifactorial origin, involving different factors related to host, parasite and drug [41]. Thus, the comprehension of resistance molecular mechanisms in *Leishmania* spp. is essential to identify novel drug targets to prevent or reverse such mechanisms. Recently, our phosphoproteomic study identified the proteins nucleoside diphosphate kinase b (NDKb) and elongation factor 2 (EF2) differently modulated in antimony-resistant *L. braziliensis* samples [34]. The results presented in this study corroborate with these data once we observed that the NDKb and EF2 proteins were more and less expressed, respectively, in the LbSbR line than the LbWTS pair. Literature data to date provide no functional analysis of these proteins in *Leishmania*. Therefore, overexpression of *NDKb* and *EF2* genes in New World *L. braziliensis* and *L. infantum* species were performed here to investigate the contribution of these genes in antimony resistance phenotype.

NDKb is a member of the NDK family which is implicated in multiple cellular processes [42]. NDKs have a key role in the purine metabolism in pathogenic parasites such as *Leishmania* spp. and *Trypanosoma* spp., which makes these enzymes potential targets for the development of new strategies for trypanosomiasis treatment [43]. NDK, which corresponds to Nm23 gene family in the human genome, can be considered a tumor metastasis suppressor [44, 45]. Furthermore, it has differential abilities to modulate tumorigenesis [46]. Here we observed that NDKb protein is more expressed in Sb^{III}-resistant *L. braziliensis* and *L. infantum* lines than in the corresponding wild-type lines. Interestingly, proteomic analysis showed that the NDK enzyme was more abundant in a benznidazole-resistant *Trypanosoma cruzi* population [47]. In our study, transfection of the *NDKb* gene in wild-type *L. braziliensis* and *L. infantum* lines led to increase of NDKb protein levels in the transfected clones when compared to their parental counterparts, as demonstrated by Western blot results. Functional assays showed that *L. braziliensis* clonal lines overexpressing this enzyme are less susceptible to Sb^{III} in relation to untransfected parasites. Nevertheless, no difference was observed in the susceptibility to antimony between the wild-type and NDKb-overexpressing *L. infantum* lines, demonstrating that this gene does not alter the Sb^{III}-resistance phenotype in this species.

In our search for possible inhibitors of NDKb enzyme, we found a drug known as lamivudine that is used in the chemotherapy of HIV and HBV diseases. According to DrugBank (www.drugbank.ca/drugs/DB00709), lamivudine is phosphorylated to active metabolites that compete for integration into DNA of the virus. These metabolites inhibit the HIV reverse transcriptase enzyme competitively and function as a terminator of DNA chain synthesis. The absence of a 3′-OH group in the incorporated nucleoside analogue stops the formation of the 5′ to 3′ phosphodiester linkage required for DNA chain elongation, and thereby, the growth of viral DNA is finished. Our study showed that overexpression of the NDKb enzyme confers resistance to lamivudine. The results propose that the parasites express the active form of this enzyme and that lamivudine probably prevents the transfer of γ-phosphoryl groups from NTP to NDP in the parasite. In addition, our results revealed that the combination lamivudine and Sb^{III} does not alter the leishmanicidal effect, suggesting that this combination is not a good strategy to be used in the leishmaniasis chemotherapy.

Attenuation of reactive oxygen species (ROS) production is an additional function proposed for secreted NDKs by pathogenic microorganisms [48]. Our results revealed that overexpression of NDKb enzyme did not alter the susceptibility of parasites to H_2O_2. These findings suggest that NDKb enzyme is not directly involved in the defense against oxidative stress in *L. braziliensis*.

Molecular mechanisms that regulate protein synthesis are essential for various biological phenomena. Protein translation is a process regulated at the initiation and elongation levels. There are diverse factors responsible for the regulation at translation elongation, but EF2 along EF1A are one of the most important enzymes which conduct the elongation cycle of protein synthesis in eukaryotic cells [49]. Our previous phosphoproteomic study demonstrated that EF2 protein presented lower abundance in Sb^{III}-resistant *L. braziliensis* samples [34], suggesting that this protein was dephosphorylated (active state) to regulate the elongation of essential proteins which are crucial to maintain the antimony resistance phenotype. Other studies also found EF2 contributing to this phenotype in different *Leishmania* species. EF2 was found with higher abundance in Sb^{V}-resistant *L. donovani* isolates [50], Sb^{III}-resistant *L. panamensis* and *L. infantum* lines [33, 51]. However, according our previous results this protein probably might be dephosphorylated in these antimony-resistant parasites. Thus, these studies demonstrate that there are differences among *Leishmania* species, which can result in variations in the degree of phosphorylation or expression of the EF2 protein.

In this work, we transfected the *EF2* gene to analyze if the overexpression of this enzyme contributes for Sb^{III} resistance in *Leishmania* species. Western blot assays

revealed that the expression level of this protein was higher in *L. braziliensis* and *L. infantum* lines overexpressing EF2 in comparison with their respective wild-type counterparts. Experiments of susceptibility to antimony showed that both *L. braziliensis* clones transfected with *EF2* gene were more resistant to Sb^{III} than the controls (wild-type and empty vector). On the other hand, the EF2-overexpressing *L. infantum* lines did not present difference in the susceptibility to Sb^{III} when compared to its parental line, showing that this gene is not relevant to Sb^{III} resistance in this *Leishmania* species. Overexpression of EF2 was observed in patients with lung adenocarcinoma [52] and related with reduced cell death after exposure to cumene hydroperoxide [53]. Kushawaha et al. [54] demonstrated that EF2, a Th1 stimulatory protein of *L. donovani*, produces strong IFN-γ and IL-12 response in cured *Leishmania*-infected patients/hamsters and confers considerable protection against experimental visceral leishmaniasis.

EF2 can be regulated through inhibitory phosphorylation at threonine 56 (T56) by EF2K [55]. Phosphorylation on T56 inactivates EF2 and it is the unique known regular EF2 functional alteration. Differently, EF2K suffers vast regulatory phosphorylations that permit distinct pathways to impact elongation [32]. Our results showed that EF2-overexpressing clone showed slight resistance to the EF2K inhibitor in comparison with the wild-type line. Surprisingly, this inhibitor increased the antileishmanial effect of Sb^{III}, especially against EF2-overexpressing parasites. Chen et al. [40] showed that the concentrations of this inhibitor that effectively inhibited EF2 phosphorylation did not produce significant inhibition of cancer cell proliferation.

Our results demonstrated that both NDKb and EF2 proteins of *Leishmania* presented approximately 60% of identity with the mammal proteins, indicating a good degree of conservation between these proteins. In this way, further studies are needed to investigate the cytotoxicity of lamivudine and EF2K inhibitor against mammalian cells.

Conclusions

Our findings represent the first study of *NDKb* and *EF2* genes overexpression in *Leishmania* species, demonstrating that these proteins are implicated in Sb^{III} resistance phenotype in *L. braziliensis*. Susceptibility assays showed that NDKb-overexpressing lines were more resistant to lamivudine, and EF2-overexpressing clone was moderately more resistant to EF2K inhibitor. In addition, our results suggest that the combined treatment EF2K inhibitor with Sb^{III} might be a good strategy to increase antileishmanial effect. Therefore, our data provided in this report bring new knowledges about resistance to Sb^{III} in *Leishmania* which can contribute to develop new strategies for leishmaniasis chemotherapy.

Abbreviations

EF2: Elongation factor 2; EF2K: Elongation factor 2 kinase; Lb: *Leishmania* (*V.*) *braziliensis*; Li: *Leishmania* (*L.*) *infantum*; NDKb: Nucleoside diphosphate kinase b; Sb^{III}: Potassium antimonyl tartrate; SbR: Sb^{III}-resistant; WTS: Wild-type susceptible

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Availability of data and materials

All data generated or analyzed during this study are included in the article.

Authors' contributions

Conceived, designed and performed the experiments, analyzed the data and helped to draft the manuscript: DSM and SMFM. Both authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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5 CONSIDERAÇÕES FINAIS

Há alguns anos o nosso grupo de pesquisa tem se dedicado ao estudo de mecanismos moleculares de resistência ao antimônio trivalente em espécies de *Leishmania* do Novo Mundo. Este estudo iniciou-se com a seleção e caracterização fenotípica das linhagens de *L. guyanensis*, *L. amazonensis*, *L. braziliensis* e *L. infantum* sensíveis e resistentes ao Sb^{III} por Liarte e Murta em 2010. Posteriormente, alguns estudos de caracterização molecular e análise funcional de genes foram realizados principalmente com *L. braziliensis*, uma espécie de grande importância epidemiológica em nosso país, que pode causar as formas cutânea e mucocutânea de leishmaniose.

A fosforilação de proteínas é um fenômeno essencial que regula complexos e diversos processos nas células eucarióticas. Desse modo, a regulação dinâmica da função de uma proteína através desta importante modificação pós-traducional é alcançada por um balanço das atividades de proteínas quinases e fosfatases em uma célula. Nesse contexto, foi utilizada a metodologia de eletroforese em gel diferencial bidimensional (2D-DIGE) que envolve a marcação de extratos proteicos com diferentes marcadores fluorescentes (Cy2, Cy3 e Cy5). Posteriormente, foi realizada a análise de espectrometria de massas para identificar as fosfoproteínas nas linhagens de *L. braziliensis* sensível e resistente ao antimônio trivalente. Essa ferramenta quantitativa foi bastante pertinente, uma vez que permitiu a identificação de 48 diferentes proteínas provenientes de cinco grupos de análises comparativas e de um número total de 116 *spots* (Fig. 8). Os resultados demonstraram que os grupos 1 (LbWTS 0 x LbWTS 0.025) e 5 (LbSbR 0.025 x LbSbR 2) devem estar associados com resposta ao estresse causado pelo tratamento com Sb^{III}. Por outro lado, os grupos 2 (LbWTS 0.025 x LbSbR 0.025), 3 (LbWTS 0 x LbSbR 0.025) e 4 (LbWTS 0.025 x LbSbR 2) estão relacionados a mecanismos de resistência à droga. Em seguida, as fosfoproteínas identificadas nesse estudo foram classificadas em sete categorias de processos biológicos de acordo com banco de dados. Os resultados revelaram que a categoria “enovelamento de proteínas/chaperonas e resposta ao estresse” está envolvida principalmente em resposta ao Sb^{III}, como observado nos grupos 1 e 5. Já as categorias “antioxidante/detoxificação”, “processos metabólicos”, “processamento de RNA/DNA” e “biossíntese de proteínas” estão moduladas no caso de resistência à droga para os demais grupos.

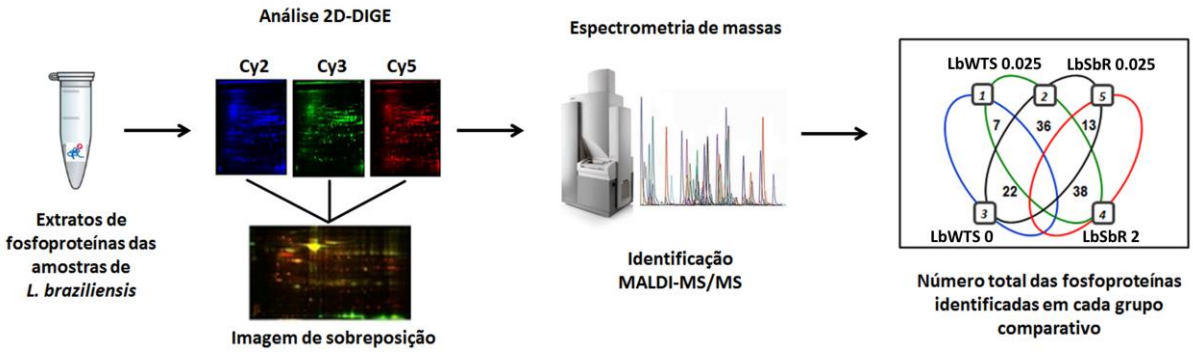

Figura 8 – Resumo gráfico da análise fosfoproteômica das linhagens de *L. braziliensis* sensível e resistente ao Sb^{III}.

As proteínas de choque térmico (HSPs) são altamente conservadas evolutivamente, agrupadas em diversas famílias e desempenham um papel fundamental na fisiologia da célula. A expressão destas proteínas não é alterada apenas devido à exposição ao calor, mas também quando as células são expostas a diferentes desafios metabólicos, como, por exemplo, em casos de pressão à droga. O efeito de exposição ao antimônio trivalente sobre a fosforilação proteica foi avaliado nas amostras de *L. braziliensis*. Os resultados da análise fosfoproteômica indicaram que duas principais proteínas de choque térmico, HSP70 e HSP83-1, apresentaram um aumento na abundância, como identificado nas análises comparativas dos grupos 1 e 5. Isso significa que o tratamento da linhagem sensível LbWTS com droga (0,025 mg/mL) e o da linhagem resistente LbSbR com alta concentração de Sb^{III} (2 mg/mL) provoca um estresse geral no parasito, que induz a produção de proteínas fosforiladas, sugerindo que as HSPs de *Leishmania* são reguladas pós-traducionalmente pela fosforilação. Este fenômeno é essencial para o parasito, pois permite que as HSPs realizem a sua principal função, que é a de atuar como chaperona molecular, além de conferir proteção para a célula. Dessa maneira, as HSPs poderão auxiliar na síntese, dobramento e degradação de proteínas enquanto o parasito estiver submetido ao estresse metabólico gerado pela droga. As análises também demonstraram que a proteína HSP70 foi identificada no grupo 2 com abundância maior na amostra LbWTS 0.025 em comparação com a amostra LbSbR 0.025, sugerindo que algumas isoformas ou modificações desta proteína estão relacionadas a mecanismos de resistência ao Sb^{III}, bem como à resposta ao tratamento com a droga.

A principal categoria de processo biológico envolvida com mecanismos de resistência a drogas é a da defesa “antioxidante/detoxificação”. As proteínas pertencentes a este sistema possuem um papel fundamental para os tripanossomatídeos. Dados desse estudo mostraram a presença de *spots* correspondentes às fosfoproteínas triparedoxina peroxidase (TXNPx) e peroxidoxina com abundância aumentada na amostra resistente LbSbR 0.025 do grupo 2. Isso demonstra que provavelmente há um aumento nos níveis de fosforilação destas proteínas, o que é essencial para a regulação da função de redução do estresse oxidativo causado pelo antimônio trivalente e, consequentemente, diminuição do dano celular. Estudo prévio realizado pelo nosso grupo de pesquisa mostrou que TXNPx está envolvida no fenótipo de resistência de *L. braziliensis* ao Sb^{III}.

Dados da literatura também relatam que as proteínas do citoesqueleto sofrem modificações pós-traducionais, dentre as quais podemos citar a fosforilação. Os resultados observados nas análises comparativas demonstraram que as proteínas alfa-tubulina, beta-tubulina e paraflagelar rod 1D apresentaram abundância diminuída na amostra LbSbR 2 do grupo 4. Este dado indica que estas proteínas provavelmente estão defosforiladas em *L. braziliensis*, controlando assim a fisiologia e/ou morfologia dos parasitos resistentes à droga.

Os resultados da análise fosfoproteômica indicaram a presença de fosfoproteínas envolvidas em diversos processos metabólicos, como metabolismo de carboidratos, proteínas, proteólise, dentre outros. As proteínas desta categoria de processo biológico apresentaram abundância diferencial nos grupos comparativos estudados. Isso revela que as proteínas identificadas podem estar fosforiladas ou defosforiladas de acordo com a necessidade do parasito. Vale a pena destacar a fosfoproteína nucleosídeo difosfato quinase b (NDKb), uma enzima envolvida na transferência de grupo fosfato de um nucleosídeo trifosfato para um nucleosídeo difosfato, que apresentou abundância aumentada nas amostras resistentes dos grupos 2, 3 e 4, demonstrando a sua associação com o fenótipo de resistência ao Sb^{III} em *L. braziliensis*. Além disso, o aumento desta proteína no fosfoproteoma do parasito deve indicar um aumento na estequiometria de fosforilação nas amostras resistentes.

Na categoria de “processamento de RNA/DNA”, foram identificadas fosfoproteínas com abundância aumentada ou diminuída nas amostras resistentes (LbSbR 0.025 ou LbSbR 2) dos grupos de análises comparativas 2, 3 e 4. Proteínas

como antígeno nuclear de proliferação celular (PCNA), proteína de ligação Ran 1 e proteína de montagem do nucleossomo estavam mais abundantes e possivelmente mais fosforiladas, sugerindo que a fosforilação destas proteínas é importante para regular as funções de síntese, replicação e reparo de DNA, modulação da cromatina e translocação de RNA nas amostras de *L. braziliensis* resistentes ao antimônio trivalente. Por outro lado, a menor abundância e eventual defosforilação das proteínas de reparo de excisão UV RAD23 e RNA helicase são essenciais para que elas realizem a sua atividade biológica nos parasitos resistentes que estão submetidos à pressão de antimônio.

As proteínas da categoria de “biossíntese de proteínas” são também reguladas pós-traducionalmente pela fosforilação. Isso é corroborado pelo aumento na abundância das fosfoproteínas argininosuccinato sintetase, fator de iniciação da tradução 1A e proteína ribossomal S12 40S nos parasitos resistentes dos grupos 2 a 4, sugerindo que estas proteínas estejam envolvidas na síntese de outras proteínas essenciais de *L. braziliensis* resistente ao Sb^{III}. Desse modo, o controle da maquinaria de biossíntese de proteínas pela fosforilação também parece ser crucial para a sobrevivência dos parasitos resistentes à droga.

As análises do fosfoproteoma de *L. braziliensis* identificaram 12 fosfoproteínas como hipotéticas. Buscas mais recentes no banco de dados TriTrypDB (http://tritrypdb.org/tritrypdb/) demonstraram que apenas quatro proteínas (LbrM.02.0520, LbrM.30.0640, LbrM.32.1870 e LbrM.35.1650) ainda estão anotadas como hipotéticas. As demais proteínas apresentam anotação específica, a saber: LbrM.13.0270 (*ALBA-domain protein 1*), LbrM.14.0460 (*N-terminal conserved domain of Nudc./CS domain containing protein, putative*), LbrM.25.1690 (*eukaryotic translation initiation factor 3 subunit j*), LbrM.29.0260 (*TPR repeat, putative*), LbrM.30.3080 (*RNA-binding motif protein 42, putative*), LbrM.30.3140 (*translocon-associated protein beta, putative*), LbrM.33.0650 (*paraflagellar rod component, putative*) e LbrM.34.4450 (*co-chaperone protein P23*). É importante ressaltar que estas proteínas desempenham funções importantes no parasito, como: associação com proteínas de ligação de RNA (LbrM.13.0270 e LbrM.30.3080), migração nuclear (LbrM.14.0460), síntese de proteínas (LbrM.25.1690), participação na interação entre proteínas e montagem de complexos multiproteicos (LbrM.29.0260), translocação de proteínas (LbrM.30.3140), composição flagelar (LbrM.33.0650) e atuação como co-chaperona de HSP90 (LbrM.34.4450). Dessa maneira, a

fosforilação também deve estar associada na regulação das funções dessas proteínas essenciais para os parasitos submetidos à pressão de droga.

Algumas fosfoproteínas foram encontradas relacionadas com a resposta ao tratamento com droga e a mecanismos de resistência ao Sb^{III}. Os resultados demonstraram que um aumento na abundância de calreticulina, uma chaperonina, deve ser importante no caso de resistência à droga, enquanto que abundância diminuída e provável defosforilação desta proteína deve estar associada à resposta ao estresse induzido pela droga. A proteína de membrana dos kinetoplastídeos-11 (KMP-11), pertencente à categoria de processo biológico "antioxidante/detoxificação", foi identificada nos grupos 1, 2 e 5. Um aumento de abundância desta proteína na amostra resistente LbSbR 0.025 do grupo 2 indica a sua importância em mecanismos de resistência ao Sb^{III}, ao passo que uma diminuição na abundância de KMP-11 nas amostras LbWTS 0.025 (grupo 1) e LbSbR 2 (grupo 5) está associada à resposta ao estresse causado pelo antimônio. É importante ressaltar que embora KMP-11 tenha sido incluída na categoria citada anteriormente através de banco de dados, esta proteína tem sido descrita na literatura como marcador de diagnóstico e antígeno vacinal. A proteína pteridina redutase 1 (PTR1), uma enzima da defesa antioxidante, foi observada com alta abundância nos parasitos resistentes LbSbR 0.025 e LbSbR 2 dos grupos 2 a 4, indicando que esta proteína também é regulada pós-traducionalmente pela fosforilação, no caso de resistência à droga. Entretanto, a diminuição na abundância desta proteína na amostra LbSbR 2 (grupo 5) está relacionada à resposta de parasitos resistentes a uma alta concentração de Sb^{III}. É importante salientar que foi realizada a análise funcional da enzima PTR1 e os resultados mostraram que esta proteína está envolvida no fenótipo de resistência de *L. braziliensis* ao antimônio trivalente. Outra proteína conhecida como fator de elongação 2 (EF2), que participa da biossíntese de proteínas, apresentou abundância aumentada nos parasitos sensíveis comparados aos resistentes (grupos 2 a 4) e maior abundância na amostra LbSbR 0.025 do que na amostra LbSbR 2 (grupo 5), demonstrando que provável diminuição na fosforilação desta proteína pode estar envolvida em mecanismos de resistência ao antimônio, mas também na resposta à alta pressão de droga.

É importante salientar que a fosforilação ou defosforilação de uma proteína não significa necessariamente que a proteína estará em seu estado ativo ou inativo,

respectivamente. Pelo contrário, a proteína pode ser inibida ou ativada através da ação de diferentes quinases e de acordo com a necessidade bioquímica da célula. Dessa forma, a fosforilação ou defosforilação pode ser vista como um mecanismo essencial que pode ser utilizado para ativar ou desativar determinadas vias com o objetivo de tolerar a situação de estresse e/ou resistência à droga em que o parasito estiver submetido.

A identificação e quantificação de sítios de fosforilação nas proteínas de tripanossomatídeos são de extrema relevância para aumentar a compreensão das vias de sinalização celular e os impactos gerados na célula. Alguns estudos demonstrados na literatura utilizaram enriquecimento dos fosfopeptídeos com dióxido de titânio para identificar e quantificar esses sítios em *Leishmania*, associando com a função biológica que desempenham no parasito. Entretanto, nessa análise fosfoproteômica não foi utilizada esta técnica de enriquecimento para obtenção dos sítios específicos de fosforilação nas amostras de *L. braziliensis* estudadas, representando assim uma limitação desse estudo. Por outro lado, análises de alinhamentos de sequências múltiplas de nove fosfoproteínas identificadas no fosfoproteoma com sequências de resíduos fosforilados, que foram relatados anteriormente na literatura em espécies de *Leishmania*, revelaram que a fosforilação é comum em resíduos de serina (S) nas espécies deste parasito. Nesse sentido, a investigação específica dos sítios de fosforilação nas amostras de *Leishmania* é essencial para analisar o perfil de fosforilação e elucidar eventos de sinalização associados à resposta ao estresse causado pelo Sb^{III} e ao fenótipo de resistência à droga. Além disso, esse tipo de análise poderá subsidiar estudos de mutagênese sítio-dirigida para avaliar a importância dos sítios fosforilados na função das proteínas do parasito, bem como na identificação de potenciais alvos quimioterápicos.

Esse estudo fosfoproteômico foi o primeiro a determinar o perfil de fosfoproteínas das amostras de *L. braziliensis* sensíveis e resistentes ao Sb^{III}, com destaque para proteínas associadas com o estresse causado pelo tratamento com droga e a mecanismos de resistência. Todavia, outra limitação deste estudo é a informação sobre a abundância e fosforilação de proteínas de membrana. Dados da literatura indicam que é necessária a utilização de protocolos específicos para obtenção de frações enriquecidas de proteínas de membrana, uma vez que este tipo de proteína apresenta baixa solubilidade em géis bidimensionais.

Os resultados obtidos com a análise fosfoproteômica poderão auxiliar na seleção de novos alvos e pesquisas que contribuam para o melhor entendimento da fosforilação e das principais vias envolvidas no fenótipo de resistência ao Sb^{III} em *Leishmania*. Este estudo ainda permitirá a análise funcional de diversas proteínas identificadas nas análises comparativas, bem como novos estudos fosfoproteômicos de outras espécies de *Leishmania*.

A segunda parte desse estudo envolveu a análise funcional das proteínas NDKb e EF2, as quais foram selecionadas a partir do fosfoproteoma de *L. braziliensis*. Como citado anteriormente, NDKb é uma enzima ubíqua fundamental para a síntese de nucleosídeos trifosfatos, além de ter um papel crucial no metabolismo de purinas em tripanossomatídeos. Nesse estudo, foi observado que a proteína NDKb está mais expressa em linhagens de *L. braziliensis* e *L. infantum* resistentes ao Sb^{III} comparadas com seus respectivos pares sensíveis. Curiosamente, dados de análise proteômica mostraram que a enzima NDK estava mais abundante em uma população de *Trypanosoma cruzi* resistente ao benzonidazol.

A enzima EF2 é um fator relevante na síntese de proteínas do parasito. Resultados do fosfoproteoma demonstraram que a proteína EF2 apresentou menor abundância em amostras de *L. braziliensis* resistentes ao Sb^{III}, sugerindo que esta proteína esteja defosforilada (estado ativo) para regular a elongação de proteínas cruciais para manter o fenótipo de resistência ao antimônio. Outros estudos também encontraram EF2 contribuindo para este fenótipo em diferentes espécies de *Leishmania*. EF2 foi encontrada com maior abundância em *L. donovani*, *L. panamensis* e *L. infantum* resistentes ao antimônio. Contudo, de acordo com os resultados anteriores, esta proteína pode estar defosforilada nestes parasitos resistentes ao antimônio. Assim, esses estudos demonstram que existem diferenças entre as espécies de *Leishmania*, podendo resultar em variações no grau de fosforilação ou expressão da proteína EF2.

Inicialmente, as enzimas NDKb e EF2 foram superexpressas em *L. braziliensis* e *L. infantum* para investigar os seus papéis no fenótipo de resistência ao antimônio trivalente nestas espécies do parasito. Ensaios de transfecção demonstraram que as linhagens de *L. braziliensis* superexpressoras de NDKb ou EF2 foram mais resistentes ao Sb^{III} em comparação aos parasitos não transfectados. Entretanto, nenhuma diferença foi observada na susceptibilidade ao

antimônio entre as linhagens de *L. infantum* superexpressoras de NDKb ou EF2 e os controles (linhagem sensível e vetor vazio). Dessa maneira, este resultado sugere que os genes *NDKb* e *EF2* não estão envolvidos diretamente no fenótipo de resistência de *L. infantum* ao antimônio trivalente.

Testes de inibição são de extrema importância para provar que as enzimas superexpressas nos parasitos são funcionais/ativas e que os inibidores utilizados apresentam atividade específica. Nesse sentido, foi realizada uma busca no DrugBank para encontrar possíveis fármacos que pudessem ser utilizados em ensaios de inibição da enzima NDKb. Os resultados apresentaram três agentes antivirais: tenofovir, lamivudina e adefovir dipivoxil. Estas drogas são utilizadas no tratamento da hepatite B crônica (HBV) e as duas primeiras são também úteis no tratamento da infecção causada pelo vírus HIV. A lamivudina é um análogo de nucleosídeo sintético que é fosforilado intracelularmente ao metabólito ativo (lamivudina trifosfato), o qual é incluído no DNA viral pela transcriptase reversa do HIV e pela polimerase do HBV, causando a terminação da cadeia de DNA. Esse estudo mostrou que os clones de *L. braziliensis* superexpressores de NDKb foram resistentes à lamivudina, sugerindo que estes parasitos expressam a forma ativa desta enzima. Entretanto, os nossos resultados revelaram que a combinação de lamivudina com o antimônio trivalente não aumenta o efeito leishmanicida, demonstrando que esta associação de drogas não deve ser uma boa estratégia a ser considerada no tratamento das leishmanioses. Uma possível explicação para a baixa atividade da lamivudina pode estar relacionada ao alto EC_{50} (concentração efetiva que inibe o crescimento em 50%) desta droga encontrado para os parasitos superexpressores da enzima NDKb em comparação com a linhagem não transfectada. É interessante destacar que a lamivudina também foi testada contra *T. cruzi* pelo Dr. Policarpo Sales Júnior da Plataforma de Bioensaios do CPqRR e os resultados indicaram que a droga também não foi ativa neste parasito.

Dados da literatura mostram que a proteína EF2 é inativada através da fosforilação da treonina 56 pela quinase de EF2 (EF2K). Análises do fosfoproteoma indicaram que EF2 provavelmente está defosforilada nas amostras de *L. braziliensis* resistentes ao antimônio, realizando a elongação de diversas proteínas nestes parasitos. Sendo assim, foram realizados ensaios para avaliar o efeito do inibidor de EF2K no crescimento das linhagens de *L. braziliensis* sensível e superexpressora de EF2. Os resultados revelaram que o clone superexpressor de EF2 foi 1,2 vezes mais

resistente ao inibidor em relação ao parasito sensível. Posteriormente, foram realizados testes de competição do inibidor de EF2K com o Sb^{III}. De maneira surpreendente, foi observada uma maior susceptibilidade no crescimento do clone de *L. braziliensis* superexpressor de EF2 ao ser exposto à combinação destas drogas, demonstrando um envolvimento da enzima EF2 nesta atividade. Este dado sugere que a associação do inibidor de EF2K com Sb^{III} possa ser considerada uma estratégia promissora para o tratamento das leishmanioses.

Os resultados também demonstraram que as proteínas NDKb e EF2 de *Leishmania* apresentaram uma identidade de cerca de 60% com as proteínas de mamíferos, indicando um bom grau de conservação entre estas proteínas. Dessa forma, experimentos adicionais serão importantes para avaliar a citotoxicidade da lamivudina e do inibidor de EF2K contra as células de mamíferos. Além disso, realizar ensaios de infecção de macrófagos e investigar a atividade destas drogas na forma amastigota de *L. braziliensis*, assim como fazer ensaios *in vivo* em modelos murinos serão essenciais para validar melhor estes inibidores na quimioterapia das leishmanioses. Outra estratégia interessante seria testar os parasitos superexpressores das enzimas NDKb e EF2 com outros fármacos utilizados no tratamento da doença, com o intuito de analisar a ocorrência de resistência cruzada. Ainda, a utilização de modelagem molecular é uma ferramenta que pode ser útil na descoberta de novos fármacos para serem avaliados nesses parasitos.

A figura 9 representa um esquema gráfico dos resultados que mostram pela primeira vez na literatura o envolvimento dos genes *NDKb* e *EF2* no fenótipo de resistência ao antimônio trivalente em *L. braziliensis*. Nesse sentido, este estudo também contribui para uma melhor compreensão dos mecanismos de resistência ao Sb^{III} em *Leishmania*.

Finalizando, os resultados obtidos com a análise fosfoproteômica e genômica funcional das linhagens de *L. braziliensis* sensível e resistente ao antimônio trivalente permitem propor um modelo de possível fosforilação ou defosforilação de determinadas vias que podem contribuir para o fenótipo de resistência ao Sb^{III} (Fig. 10). Neste modelo, o aumento na fosforilação de importantes enzimas da defesa antioxidante, como triparedoxina peroxidase (TXNPx), peroxidoxina, pteridina redutase 1 (PTR1) e proteína de membrana dos kinetoplastídeos-11 (KMP-11) contribui para a detoxificação das espécies reativas de oxigênio (EROs) produzidas pelo Sb^{III}, fornecendo proteção contra o estresse oxidativo e evitando a morte do

parasito. Outro mecanismo de ação do antimonial está associado à morte celular através da fragmentação de DNA. Nesse contexto, a fosforilação da proteína antígeno nuclear de proliferação celular (PCNA) e a defosforilação da enzima de reparo de excisão UV RAD 23 estariam envolvidas na resistência ao antimônio trivalente por meio da síntese e reparo de DNA. O aumento na fosforilação de argininosuccinato sintetase (ASS), fator de iniciação da tradução 1A e da proteína ribossomal S12 40S é de extrema importância para a participação destas enzimas na síntese de diversas proteínas que contribuem para a manutenção da resistência ao Sb^{III} em *L. braziliensis*. Além disso, a defosforilação do fator de elongação 2 (EF2), que é útil no crescimento da cadeia polipeptídica, e a fosforilação da proteína de choque térmico HSP83-1, uma chaperonina envolvida no enovelamento de proteínas, são também cruciais quando a maquinaria de produção proteica é ativada pelo parasito resistente. A defosforilação de proteínas do citoesqueleto (α e β-tubulinas) é outra via regulada pós-traducionalmente, que é essencial para a sustentação do processo de divisão celular, transporte intracelular e motilidade flagelar dos parasitos submetidos à pressão da droga. A fosforilação da enzima nucleosídeo difosfato quinase b (NDKb) também é relevante para manter a produção de nucleosídeos trifosfatos (NTPs), os quais são essenciais em todos os processos celulares que envolvem a síntese de ácidos nucleicos em *L. braziliensis* resistente ao Sb^{III}.

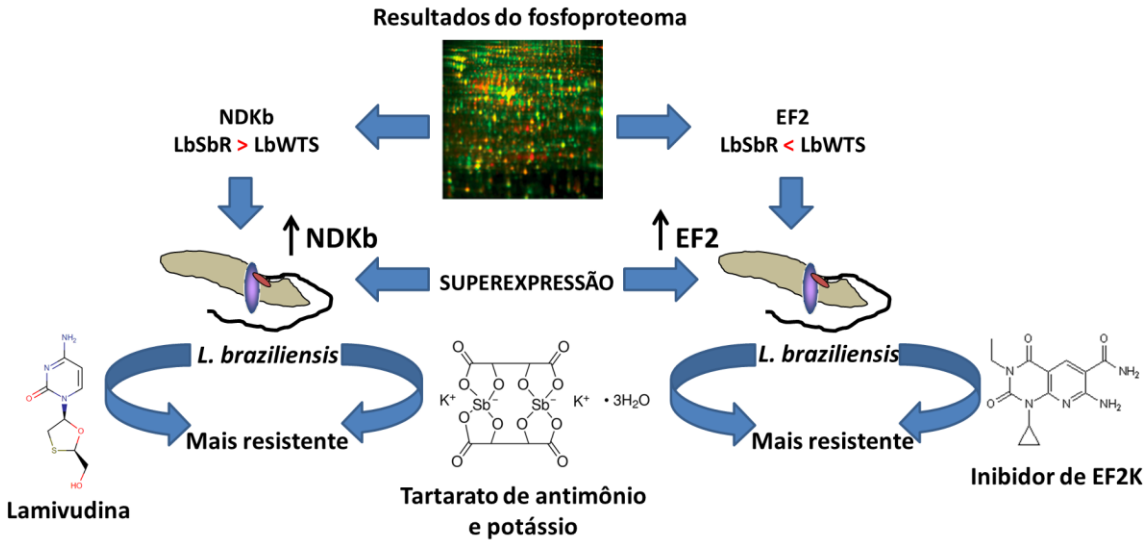

Figura 9 – Resumo gráfico da análise funcional dos genes *NDKb* e *EF2* em *L. braziliensis*.

Portanto, uma variedade de mecanismos de resistência desenvolvidos por diversos organismos tem sido descrita na literatura. Entretanto, a resistência a drogas, especialmente em *Leishmania*, continua sendo um grande desafio a ser desvendado pelos pesquisadores. Estes estudos continuam sendo de extrema importância para a identificação de novos alvos que possam ser utilizados em novas abordagens quimioterapêuticas contra as leishmanioses. Dessa forma, esse estudo pode contribuir para a elucidação de possíveis mecanismos de resistência ao antimônio trivalente em *Leishmania*, servindo de base para outras pesquisas científicas.

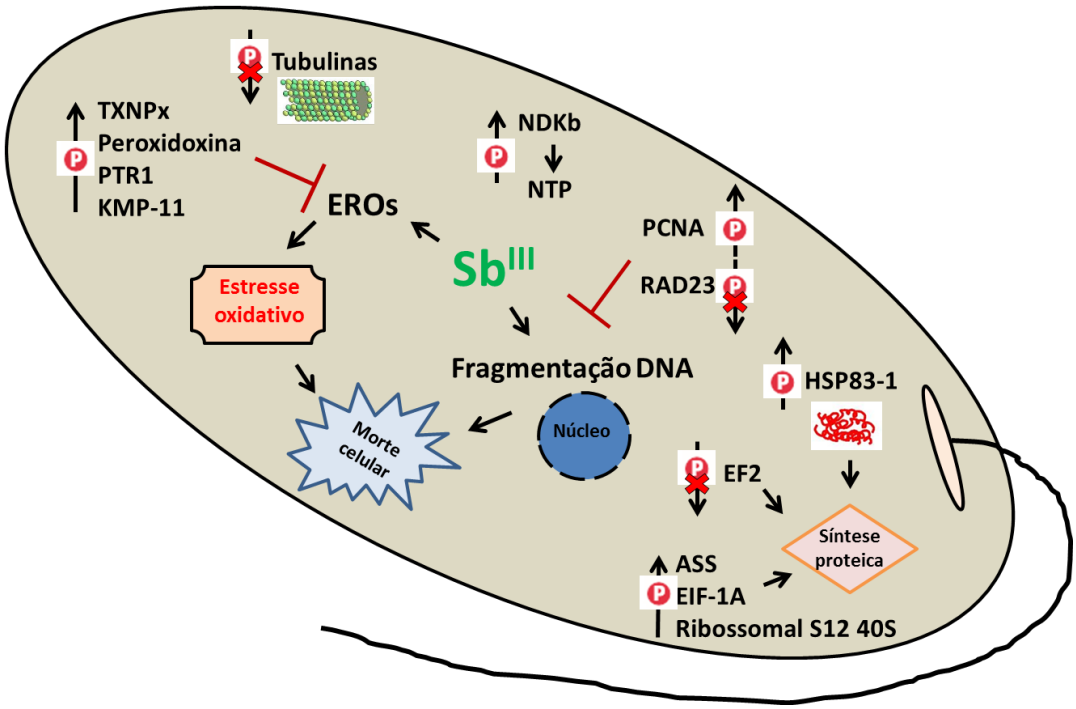

Figura 10 – Modelo proposto de possíveis processos regulados pela fosforilação com impacto na resistência ao Sb^{III} em *L. braziliensis*. EROs (espécies reativas de oxigênio), TXNPx (triparedoxina peroxidase), PTR1 (pteridina redutase 1), KMP-11 (proteína de membrana dos kinetoplastídeos-11), EF2 (fator de elongação 2), ASS (argininosuccinato sintetase), EIF-1A (fator de iniciação da tradução 1A), HSP83-1 (proteína de choque térmico 83-1), PCNA (antígeno nuclear de proliferação celular), RAD23 (proteína de reparo de excisão UV RAD23), NDKb (nucleosídeo difosfato quinase b), NTP (nucleosídeo trifosfato), P indica fosforilado e P marcado com um X vermelho significa defosforilado.

6 RESUMO DOS RESULTADOS

A partir desse estudo de análise fosfoproteômica e genômica funcional das linhagens de *Leishmania* spp. sensíveis e resistentes ao Sb^{III}, pode-se destacar os seguintes resultados alcançados:

- ✓ Análises comparativas demonstraram a presença de 116 *spots* diferencialmente abundantes entre os cinco grupos analisados, incluindo 11 e 34 *spots* especificamente correlacionados ao tratamento com droga e à resistência ao Sb^{III}, respectivamente;
- ✓ Foram identificadas 48 diferentes fosfoproteínas distribuídas em sete categorias de processos biológicos;
- ✓ Análises *in silico* demonstraram a conservação, principalmente, de resíduos de serina fosforilados nas nove proteínas analisadas;
- ✓ Ensaios de Western blot revelaram níveis de expressão diferencial das fosfoproteínas cTXNPx, KMP-11 e α-tubulina nas amostras de *L. braziliensis* estudadas, validando os resultados fosfoproteômicos;
- ✓ As linhagens de *L. braziliensis* superexpressoras de NDKb ou EF2 foram mais resistentes ao Sb^{III} do que a linhagem sensível;
- ✓ Nenhuma diferença na susceptibilidade ao Sb^{III} foi observada nas linhagens de *L. infantum* superexpressoras de NDKb ou EF2 comparadas com os controles;
- ✓ As linhagens de *L. braziliensis* superexpressoras de NDKb apresentaram elevada resistência à lamivudina, que não alterou a atividade leishmanicida ao ser associada com o Sb^{III};
- ✓ O clone de *L. braziliensis* superexpressor de EF2 foi 1,2 vezes mais resistente ao inibidor de EF2K, que apresentou maior efeito leishmanicida em combinação com o Sb^{III};
- ✓ As linhagens de *L. braziliensis* superexpressoras de NDKb não alteraram a sua susceptibilidade ao H_2O_2;
- ✓ Análises de Western blot revelaram que as proteínas NDKb e EF2 estão mais e menos expressas, respectivamente, na linhagem de *L. braziliensis* resistente ao Sb^{III}.

7 CONCLUSÃO FINAL

A análise fosfoproteômica permitiu demonstrar pela primeira vez o perfil das fosfoproteínas diferencialmente abundantes em *L. braziliensis*, fornecer novos conhecimentos sobre mecanismos pós-traducionais associados com resposta ao estresse causado pelo tratamento com droga e resistência ao antimônio trivalente, além de compreender melhor a biologia de *Leishmania*. Além disso, a análise funcional dos genes *NDKb* e *EF2* representa o primeiro estudo que demonstrou a superexpressão dessas proteínas e o envolvimento delas com o fenótipo de resistência ao Sb^{III} em *L. braziliensis*. Dessa maneira, esse estudo contribui com novos dados para o campo de resistência a drogas em *Leishmania*, proporcionando o desenvolvimento de outros estudos na área e de novas intervenções quimioterapêuticas para serem utilizadas no controle das leishmanioses.

8 PERSPECTIVAS

- ✓ Realizar a análise funcional de outras proteínas identificadas nesse estudo;
- ✓ Identificar os sítios de fosforilação e analisar o perfil de fosforilação das proteínas de *L. braziliensis*;
- ✓ Fazer mutagênese sítio-dirigida dos sítios de fosforilação de algumas proteínas;
- ✓ Realizar ensaios de Western blot para validar outras proteínas identificadas na análise fosfoproteômica;
- ✓ Avaliar a atividade anti-*Leishmania* da lamivudina e do inibidor de EF2K em ensaios *in vitro*, utilizando as formas amastigotas do parasito, e *in vivo* em modelos murinos.

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APÊNDICES

I – Alinhamento da sequência de nucleotídeos obtida por sequenciamento com a sequência referência do gene *NDKb* depositada no TriTrypDB (LbrM.32.3210)

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> LbrM.32.3210 | organism=Leishmania_braziliensis_MHOM/BR/75/M2904
| product=nucleoside diphosphate kinase b | location=LbrM.32:1182666-1183121(-)
| length=456 | sequence_SO=chromosome
| SO=protein_coding
Length=456

 Score =  823 bits (912),  Expect = 0.0
 Identities = 456/456 (100%), Gaps = 0/456 (0%)
 Strand=Plus/Plus

Query  1    ATGTCCTCCGAGCGCACTTTCATTGCCATCAAGCCGGACGGTGTTCAGCGCGGCCTCGTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGTCCTCCGAGCGCACTTTCATTGCCATCAAGCCGGACGGTGTTCAGCGCGGCCTCGTT  60

Query  61   GGCGAGATCATCAGCCGCTTTGAGCGCAAGGGCTTCAAGCTTGTCGCCTTGAAGATGCTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGCGAGATCATCAGCCGCTTTGAGCGCAAGGGCTTCAAGCTTGTCGCCTTGAAGATGCTG  120

Query  121  CAGCCGACGACGGAGCAGGCCCAGGGTCACTATAAGGACCTTGCCTCCAAGCCGTTCTTC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CAGCCGACGACGGAGCAGGCCCAGGGTCACTATAAGGACCTTGCCTCCAAGCCGTTCTTC  180

Query  181  GAGGGTCTTGTGAAGTACTTCTCGTCTGGCCCTATTGTGTGCATGGTCTGGGAGGGTAAG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GAGGGTCTTGTGAAGTACTTCTCGTCTGGCCCTATTGTGTGCATGGTCTGGGAGGGTAAG  240

Query  241  AACGTGGTGAAGAGCGGCCGTGTGTTGCTCGGCGCGACGAACCCGGCCGACTCGCAGCCC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AACGTGGTGAAGAGCGGCCGTGTGTTGCTCGGCGCGACGAACCCGGCCGACTCGCAGCCC  300

Query  301  GGCACGATCCGTGGCGACTACGCCGTGGATGTGGGCCGAAACGTGTGCCACGGCTCCGAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GGCACGATCCGTGGCGACTACGCCGTGGATGTGGGCCGAAACGTGTGCCACGGCTCCGAC  360

Query  361  TCCGTGGAGAGCGCGCAGCGCGAGGTCGCCTTCTGGTTCAAGGTGGAGGAGATCGCAAGC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TCCGTGGAGAGCGCGCAGCGCGAGGTCGCCTTCTGGTTCAAGGTGGAGGAGATCGCAAGC  420

Query  421  TGGACGTCGCATTCCGCTTGCCAGATCTACGAATAG  456
            ||||||||||||||||||||||||||||||||||||
Sbjct  421  TGGACGTCGCATTCCGCTTGCCAGATCTACGAATAG  456
```

II – Alinhamento da sequência de nucleotídeos obtida por sequenciamento com a sequência referência do gene *EF2* depositada no TriTrypDB (LbrM.35.0270)

```
> LbrM.35.0270 | organism=Leishmania_braziliensis_MHOM/BR/75/M2904
| product=elongation factor 2 | location=LbrM.35:84057-86594(-)
| length=2538 | sequence_SO=chromosome | SO=protein_coding
Length=2538

 Score = 4578 bits (5076),  Expect = 0.0
 Identities = 2538/2538 (100%), Gaps = 0/2538 (0%)
 Strand=Plus/Plus

Query  1     ATGGTGAACTTTACCGTCGATCAGGTCCGTGAGCTGATGGACTTCCCGGACCAGATCCGG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     ATGGTGAACTTTACCGTCGATCAGGTCCGTGAGCTGATGGACTTCCCGGACCAGATCCGG  60

Query  61    AACATGTCCGTGATTGCCCACGTCGACCACGGCAAGTCGACACTGTCTGACTCTCTCGTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    AACATGTCCGTGATTGCCCACGTCGACCACGGCAAGTCGACACTGTCTGACTCTCTCGTT  120

Query  121   GGCGCCGCTGGCATCATCAAGATGGAGGAGGCTGGCGACAAGCGTATCATGGATACGCGC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GGCGCCGCTGGCATCATCAAGATGGAGGAGGCTGGCGACAAGCGTATCATGGATACGCGC  180

Query  181   GCGGATGAGATTGCGCGTGGTATCACGATCAAGTCCACCGCCATCTCCATGCACTACCAC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   GCGGATGAGATTGCGCGTGGTATCACGATCAAGTCCACCGCCATCTCCATGCACTACCAC  240

Query  241   GTGCCGAAGGAGATGATTAGCAGCCTGGATGACGACAAGCGCGACTTCCTGATCAACCTG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   GTGCCGAAGGAGATGATTAGCAGCCTGGATGACGACAAGCGCGACTTCCTGATCAACCTG  300

Query  301   ATCGACTCCCCCGGACACGTCGACTTCAGCTCCGAGGTGACTGCCGCTCTTCGTGTGACG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   ATCGACTCCCCCGGACACGTCGACTTCAGCTCCGAGGTGACTGCCGCTCTTCGTGTGACG  360

Query  361   GACGGTGCGCTGGTCGTGGTGGACTGTGTGGAGGGCGTGTGCGTGCAGACGGAGACGGTG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   GACGGTGCGCTGGTCGTGGTGGACTGTGTGGAGGGCGTGTGCGTGCAGACGGAGACGGTG  420

Query  421   CTGCGCCAGGCGCTGACGGAGCGTATCCGCCCTGTTGTGTTCATCAACAAGGTGGACCGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CTGCGCCAGGCGCTGACGGAGCGTATCCGCCCTGTTGTGTTCATCAACAAGGTGGACCGC  480

Query  481   GCCATCCTTGAGCTCCAGCTGGACCCCGAAGAGGCATACCAGGGCTTCGTGAAGACGCTG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GCCATCCTTGAGCTCCAGCTGGACCCCGAAGAGGCATACCAGGGCTTCGTGAAGACGCTG  540

Query  541   CAGAACGTGAATGTGGTGGTTGCCACGTACAATGATCCCAGCATGGGGGACGTGCAGGTG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   CAGAACGTGAATGTGGTGGTTGCCACGTACAATGATCCCAGCATGGGGGACGTGCAGGTG  600

Query  601   TCGCCCGAGAAGGGCACTGTGGCGATCGGCTCTGGTCTGCAGGCGTGGGCGTTCTCGCTG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TCGCCCGAGAAGGGCACTGTGGCGATCGGCTCTGGTCTGCAGGCGTGGGCGTTCTCGCTG  660

Query  661   ACCCGCTTCGCGAACATGTATGCGTCGAAGTTCGGCGTGGACGAGCTGAAGATGCGCGAG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   ACCCGCTTCGCGAACATGTATGCGTCGAAGTTCGGCGTGGACGAGCTGAAGATGCGCGAG  720

Query  721   CGTCTGTGGGGCGACAACTTCTTTGACGCGAAGAACAAGAAGTGGATCAAGCAGGAGACG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   CGTCTGTGGGGCGACAACTTCTTTGACGCGAAGAACAAGAAGTGGATCAAGCAGGAGACG  780

Query  781   AACGCCGATGGCGAGCGCGTGCGCCGCGCGTTCTGCCAGTTCTGCCTGGACCCCATCTAC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   AACGCCGATGGCGAGCGCGTGCGCCGCGCGTTCTGCCAGTTCTGCCTGGACCCCATCTAC  840

Query  841   CAGATCTTCGACGCTGTGATGAACGAGAAGAAGGACAAGGTGGACAAGATGCTCAAGTCG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   CAGATCTTCGACGCTGTGATGAACGAGAAGAAGGACAAGGTGGACAAGATGCTCAAGTCG  900

Query  901   CTGCACGTGTCGCTGACGGCTGAGGAGCGCGAGCAGGTGCCGAAGAAGCTGCTGAAGACG  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   CTGCACGTGTCGCTGACGGCTGAGGAGCGCGAGCAGGTGCCGAAGAAGCTGCTGAAGACG  960

Query  961   GTGATGATGAGGTTTCTGCCGGCCGCTGAGACGCTGCTGCAGATGATCGTGGCGCACCTG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   GTGATGATGAGGTTTCTGCCGGCCGCTGAGACGCTGCTGCAGATGATCGTGGCGCACCTG  1020

Query  1021  CCGTCGCCCAAGAGAGCGCAGGCGTACCGCGCGGAGATGTTGTACTCTGGTGAGGCGTCG  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  CCGTCGCCCAAGAGAGCGCAGGCGTACCGCGCGGAGATGTTGTACTCTGGTGAGGCGTCG  1080

Query  1081  CCGGAGGACAAGTACTTCATGGGTATCAAGAACTGCGACCCCGCTGCGCCGCTCATGCTG  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  CCGGAGGACAAGTACTTCATGGGTATCAAGAACTGCGACCCCGCTGCGCCGCTCATGCTG  1140

Query  1141  TACATCAGCAAGATGGTGCCGACGGCCGACCGCGGCCGCTTCTTCGCTTTTGGCCGCATC  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  TACATCAGCAAGATGGTGCCGACGGCCGACCGCGGCCGCTTCTTCGCTTTTGGCCGCATC  1200
```

```
Query  1201  TTCTCGGGTAAGGTGCGCAGCGGCCAGAAGGTGCGGATCATGGGCAACAACTACATCTAC  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  TTCTCGGGTAAGGTGCGCAGCGGCCAGAAGGTGCGGATCATGGGCAACAACTACATCTAC  1260

Query  1261  GGCAAGAAGCAGGACCTGTACGATGACAAGCCTGTGCAGCGCTCTGTGCTGATGATGGGC  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1261  GGCAAGAAGCAGGACCTGTACGATGACAAGCCTGTGCAGCGCTCTGTGCTGATGATGGGC  1320

Query  1321  CGCTACCAGGAGGCTGTGGAGGACATGCCGTGCGGTAACGTGGTGGGCCTTGTGGGCGTG  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1321  CGCTACCAGGAGGCTGTGGAGGACATGCCGTGCGGTAACGTGGTGGGCCTTGTGGGCGTG  1380

Query  1381  GACAAGTACATCGTGAAGTCTGCGACGATCACGGACGACGGCGAGAACCCGTACCCGCTG  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1381  GACAAGTACATCGTGAAGTCTGCGACGATCACGGACGACGGCGAGAACCCGTACCCGCTG  1440

Query  1441  CGCGATATGAAGTACTCCGTGTCGCCTGTGGTGCGTGTGGCCGTGGAGGCGAAGAACCCG  1500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1441  CGCGATATGAAGTACTCCGTGTCGCCTGTGGTGCGTGTGGCCGTGGAGGCGAAGAACCCG  1500

Query  1501  TCCGACCTGCCGAAACTTGTGGAGGGCCTGAAGCGCCTTGCCAAGTCCGACCCGCTGGTG  1560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1501  TCCGACCTGCCGAAACTTGTGGAGGGCCTGAAGCGCCTTGCCAAGTCCGACCCGCTGGTG  1560

Query  1561  GTGTGCAGCATTGAGGAGTCTGGCGAGCACATCGTTGCCGGCGCTGGTGAGCTGCATCTT  1620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1561  GTGTGCAGCATTGAGGAGTCTGGCGAGCACATCGTTGCCGGCGCTGGTGAGCTGCATCTT  1620

Query  1621  GAGATTTGCCTGAAGGACCTCCAGGAGGACTTCATGAACGGTGCGCCGCTGAAGATCTCC  1680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1621  GAGATTTGCCTGAAGGACCTCCAGGAGGACTTCATGAACGGTGCGCCGCTGAAGATCTCC  1680

Query  1681  GAGCCGGTGGTGTCGTTCCGCGAGACCGTGACGGACGTGTCGTCGCAGCAGTGTCTGTCG  1740
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1681  GAGCCGGTGGTGTCGTTCCGCGAGACCGTGACGGACGTGTCGTCGCAGCAGTGTCTGTCG  1740

Query  1741  AAGTCTGCGAACAAGCACAACCGTCTGTTCTGCCGCGGTGCGCCGCTGACAGAGGAGCTT  1800
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1741  AAGTCTGCGAACAAGCACAACCGTCTGTTCTGCCGCGGTGCGCCGCTGACAGAGGAGCTT  1800

Query  1801  GCGCTGGCGATGGAGGAGGGCACCGCTGGTCCCGAGGCCGATCCGAAGGTGCGCGCGCGC  1860
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1801  GCGCTGGCGATGGAGGAGGGCACCGCTGGTCCCGAGGCCGATCCGAAGGTGCGCGCGCGC  1860

Query  1861  TTCCTTGCCGACAACTACGAGTGGGACGTGCAGGAGGCCCGCAAGATCTGGTGCTACGGC  1920
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1861  TTCCTTGCCGACAACTACGAGTGGGACGTGCAGGAGGCCCGCAAGATCTGGTGCTACGGC  1920

Query  1921  CCGGACAACCGCGGCCCGAACGTGGTCGTGGATGTGACGAAGGGTGTCCAGAACATGGGT  1980
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1921  CCGGACAACCGCGGCCCGAACGTGGTCGTGGATGTGACGAAGGGTGTCCAGAACATGGGT  1980

Query  1981  GAGATGAAGGACTCCTTTGTTGCGGCGTGGCAGTGGGCGACCCGCGAGGGTGTGCTCTGC  2040
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1981  GAGATGAAGGACTCCTTTGTTGCGGCGTGGCAGTGGGCGACCCGCGAGGGTGTGCTCTGC  2040

Query  2041  GACGAGAACATGCGCGGTGTGCGCGTGAACGTGGAGGATGTGACGATGCACGCGGACGCC  2100
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2041  GACGAGAACATGCGCGGTGTGCGCGTGAACGTGGAGGATGTGACGATGCACGCGGACGCC  2100

Query  2101  ATTCACCGTGGTGGCGGCCAGATCATCCCGACGGCGCGCCGTGTGTTCTACGCGTGCTGC  2160
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2101  ATTCACCGTGGTGGCGGCCAGATCATCCCGACGGCGCGCCGTGTGTTCTACGCGTGCTGC  2160

Query  2161  CTGACGGCGTCGCCGCGCCTGATGGAGCCGATGTTCGTGGTGGATATCCAGACCGTGGAG  2220
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2161  CTGACGGCGTCGCCGCGCCTGATGGAGCCGATGTTCGTGGTGGATATCCAGACCGTGGAG  2220

Query  2221  CACGCCATGGGCGGCATCTACGGTGTGCTGACCCGCCGCCGTGGTGTGATCATTGGCGAG  2280
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2221  CACGCCATGGGCGGCATCTACGGTGTGCTGACCCGCCGCCGTGGTGTGATCATTGGCGAG  2280

Query  2281  GAGAACCGCCCGGGCACGCCCATCTACAACGTGCGCGCGTACCTGCCGGTTGCGGAGTCG  2340
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2281  GAGAACCGCCCGGGCACGCCCATCTACAACGTGCGCGCGTACCTGCCGGTTGCGGAGTCG  2340

Query  2341  TTCGGCTTCACTGCCGACCTGCGCGCCGGAACTGGCGGCCAGGCCTTCCCGCAGTGCGTG  2400
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2341  TTCGGCTTCACTGCCGACCTGCGCGCCGGAACTGGCGGCCAGGCCTTCCCGCAGTGCGTG  2400

Query  2401  TTCGACCACTGGCAGGAGTACCCCGGTGACCCGCTAGAGACCAAGTCGCTGGCCAACGCG  2460
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2401  TTCGACCACTGGCAGGAGTACCCCGGTGACCCGCTAGAGACCAAGTCGCTGGCCAACGCG  2460

Query  2461  ACAACGCTTGCCATCCGCATGCGCAAGGGTCTGAAGCCGGAGATCCCCGGCCTGGACCAG  2520
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2461  ACAACGCTTGCCATCCGCATGCGCAAGGGTCTGAAGCCGGAGATCCCCGGCCTGGACCAG  2520

Query  2521  TTCATGGATAAATTGTAA  2538
             ||||||||||||||||||
Sbjct  2521  TTCATGGATAAATTGTAA  2538
```

III – Alinhamento das sequências de nucleotídeos do gene *NDKb* de *L. braziliensis* e *L. infantum*

```
> LinJ.32.3110 | organism=Leishmania_infantum_JPCM5 | product=nucleoside
diphosphate kinase b | location=LinJ.32:1163891-1164346(-)
| length=456 | sequence_SO=chromosome | SO=protein_coding
Length=456

 Score =  628 bits (696),  Expect = 5e-179
 Identities = 413/456 (91%), Gaps = 0/456 (0%)
 Strand=Plus/Plus

Query  1    ATGTCCTCCGAGCGCACTTTCATTGCCATCAAGCCGGACGGTGTTCAGCGCGGCCTCGTT  60
            ||||||||||||||||| || |||||| |||||||||||| || ||||||||||||||||
Sbjct  1    ATGTCCTCCGAGCGCACCTTTATTGCCGTCAAGCCGGACGGCGTGCAGCGCGGCCTCGTT  60

Query  61   GGCGAGATCATCAGCCGCTTTGAGCGCAAGGGCTTCAAGCTTGTCGCCTTGAAGATGCTG  120
            ||||||||||||  |||||| |||||||| |||| |||||| ||||||||||||||| |||
Sbjct  61   GGCGAGATCATCGCCCGCTTCGAGCGCAAAGGCTACAAGCTCGTCGCCTTGAAGATACTG  120

Query  121  CAGCCGACGACGGAGCAGGCCCAGGGTCACTATAAGGACCTTGCCTCCAAGCCGTTCTTC  180
            |||||||| ||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  121  CAGCCGACAACGGAGCAGGCCCAGGGTCACTATAAGGACCTTTCCTCCAAGCCGTTCTTC  180

Query  181  GAGGGTCTTGTGAAGTACTTCTCGTCTGGCCCTATTGTGTGCATGGTCTGGGAGGGTAAG  240
              ||  |||||||||||||||| |||||||| || ||||| ||||| ||||||||||||
Sbjct  181  CCGGCCCTTGTGAAGTACTTCTCCTCTGGCCCGATCGTGTGTATGGTGTGGGAGGGTAAG  240

Query  241  AACGTGGTGAAGAGCGGCCGTGTGTTGCTCGGCGCGACGAACCCGGCCGACTCGCAGCCC  300
            |||||||||||||||||| ||| |||||||||||||||||||| ||||| |||||
Sbjct  241  AACGTGGTGAAGAGCGGCCGCGTGCTGCTCGGCGCGACGAACCCGGCGGACTCACAGCCT  300

Query  301  GGCACGATCCGTGGCGACTACGCCGTGGATGTGGGCCGAAACGTGTGCCACGGCTCCGAC  360
            |||||||||||  |||||||  |||||||||||||||| ||||||||||||||||||||
Sbjct  301  GGCACGATCCGCGGCGACTTTGCCGTGGATGTGGGCCGCAACGTGTGCCACGGCTCCGAC  360

Query  361  TCCGTGGAGAGCGCGCAGCGCGAGGTCGCCTTCTGGTTCAAGGTGGAGGAGATCGCAAGC  420
            ||||||||||||||| |||||||| ||||||| |||||||||| ||| |||||||| |||
Sbjct  361  TCCGTGGAGAGCGCGGAGCGCGAGATCGCCTTTTGGTTCAAGGCGGATGAGATCGCGAGC  420

Query  421  TGGACGTCGCATTCCGCTTGCCAGATCTACGAATAG  456
            |||||||||||  ||||  | ||||||||||||  |||
Sbjct  421  TGGACGTCGCACTCCGTGTCCCAGATCTACGAGTAG  456
```

IV – Alinhamento das sequências de nucleotídeos do gene *EF2* de *L. braziliensis* e *L. infantum*

```
> LinJ.36.0190 | organism=Leishmania_infantum_JPCM5 | product=elongation
factor 2 (EF2-1) | location=LinJ.36:48610-51147(-)
| length=2538 | sequence_SO=chromosome | SO=protein_coding
Length=2538

 Score = 4158 bits (4610),  Expect = 0.0
 Identities = 2445/2538 (96%), Gaps = 0/2538 (0%)
 Strand=Plus/Plus

Query  1     ATGGTGAACTTTACCGTCGATCAGGTCCGTGAGCTGATGGACTTCCCGGACCAGATCCGG  60
             ||||||||||||||||||||||||||||| ||||||||||||  ||||||||||||||||
Sbjct  1     ATGGTGAACTTTACCGTCGATCAGGTCCGCGAGCTGATGGACTATCCGGACCAGATCCGG  60

Query  61    AACATGTCCGTGATTGCCCACGTCGACCACGGCAAGTCGACACTGTCTGACTCTCTCGTT  120
             ||||||||||||||||| ||||||||||||||||||||| ||||| ||||||||||||
Sbjct  61    AACATGTCCGTGATTGCTCACGTCGACCACGGCAAGTCGACGCTGTCCGACTCTCTCGTT  120

Query  121   GGCGCCGCTGGCATCATCAAGATGGAGGAGGCTGGCGACAAGCGTATCATGGATACGCGC  180
             || || || ||||||||||||||||||||||| ||||| ||||| ||||||||||||||||
Sbjct  121   GGTGCTGCCGGCATCATCAAGATGGAGGAGGCCGGCGATAAGCGGATCATGGATACGCGC  180

Query  181   GCGGATGAGATTGCGCGTGGTATCACGATCAAGTCCACCGCCATCTCCATGCACTACCAC  240
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   GCGGATGAGATCGCGCGTGGTATCACGATCAAGTCCACCGCCATCTCCATGCACTACCAC  240

Query  241   GTGCCGAAGGAGATGATTAGCAGCCTGGATGACGACAAGCGCGACTTCCTGATCAACCTG  300
             |||||||||||||||||| ||    ||||||||||||||||||||||||||||||||||||
Sbjct  241   GTGCCGAAGGAGATGATTGGCGATCTGGATGACGACAAGCGCGACTTCCTGATCAACCTG  300

Query  301   ATCGACTCCCCCGGACACGTCGACTTCAGCTCCGAGGTGACTGCCGCTCTTCGTGTGACG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   ATCGACTCCCCCGGACACGTCGACTTCAGCTCCGAGGTGACTGCCGCTCTTCGTGTGACG  360

Query  361   GACGGTGCGCTGGTCGTGGTGGACTGTGTGGAGGGCGTGTGCGTGCAGACGGAGACGGTG  420
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   GACGGCGCGCTGGTCGTGGTGGACTGCGTGGAGGGCGTGTGCGTGCAGACGGAGACGGTG  420

Query  421   CTGCGCCAGGCGCTGACGGAGCGTATCCGCCCTGTTGTGTTCATCAACAAGGTGGACCGC  480
             ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  421   CTGCGCCAGGCGCTGACGGAGCGCATCCGCCCTGTTGTGTTCATCAACAAGGTGGACCGC  480

Query  481   GCCATCCTTGAGCTCCAGCTGGACCCCGAAGAGGCATACCAGGGCTTCGTGAAGACGCTG  540
             |||||||||||||||| |||||||||| ||||| ||||||||||||||||||||||||||
Sbjct  481   GCCATCCTTGAGCTCCAACTGGACCCCGAGGAGGCGTACCAGGGCTTCGTGAAGACGCTG  540

Query  541   CAGAACGTGAATGTGGTGGTTGCCACGTACAATGATCCCAGCATGGGGGACGTGCAGGTG  600
             |||||||||| |||||||||||||||||||||||| |||||||||| ||||||||||||
Sbjct  541   CAGAACGTGAACGTGGTGGTTGCCACGTACAATGACCCCAGCATGGGCGACGTGCAGGTG  600

Query  601   TCGCCCGAGAAGGGCACTGTGGCGATCGGCTCTGGTCTGCAGGCGTGGGCGTTCTCGCTG  660
             || ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  601   TCCCCCGAGAAGGGCACTGTGGCGATCGGCTCTGGCCTGCAGGCGTGGGCGTTCTCGCTG  660

Query  661   ACCCGCTTCGCGAACATGTATGCGTCGAAGTTCGGCGTGGACGAGCTGAAGATGCGCGAG  720
             |||||||| ||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  661   ACCCGCTTTGCGAACATGTATGCGGCGAAGTTCGGCGTGGACGAGCTGAAGATGCGCGAG  720

Query  721   CGTCTGTGGGGCGACAACTTCTTTGACGCGAAGAACAAGAAGTGGATCAAGCAGGAGACG  780
             || |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  721   CGCCTGTGGGGCGACAACTTCTTCGACGCGAAGAACAAGAAGTGGATCAAGCAGGAGACG  780

Query  781   AACGCCGATGGCGAGCGCGTGCGCCGCGCGTTCTGCCAGTTCTGCCTGGACCCCATCTAC  840
             |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  781   AACGCCGATGGCGAGCGCGTGCGCCGCGCGTTCTGCCAGTTCTGCCTAGACCCCATCTAC  840

Query  841   CAGATCTTCGACGCTGTGATGAACGAGAAGAAGGACAAGGTGGACAAGATGCTCAAGTCG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   CAGATCTTCGACGCTGTGATGAACGAGAAGAAGGACAAGGTGGACAAGATGCTCAAGTCG  900

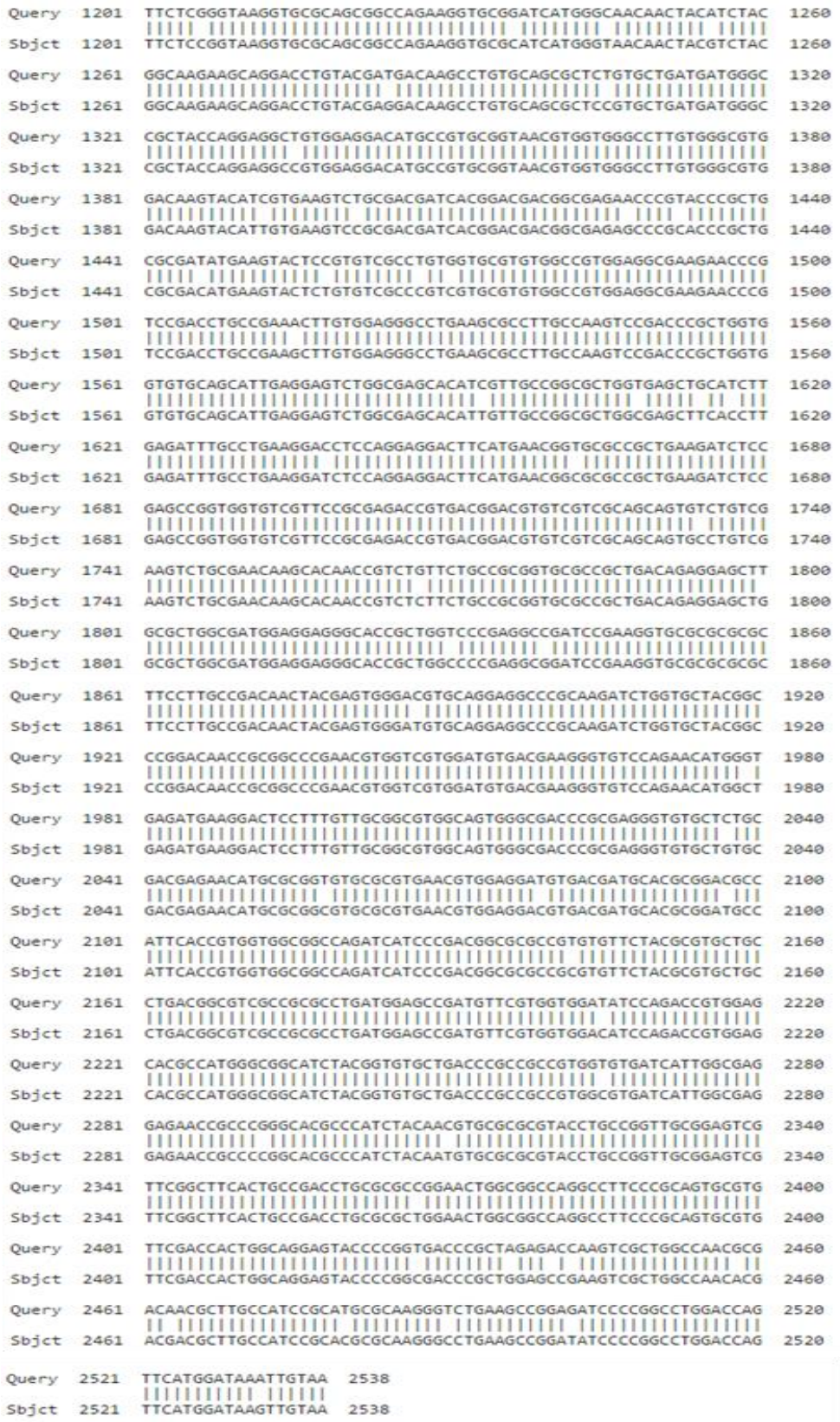
Query  901   CTGCACGTGTCGCTGACGGCTGAGGAGCGCGAGCAGGTGCCGAAGAAGCTGCTGAAGACG  960
             ||||||||| ||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  901   CTGCACGTGACGCTGACGGCTGAGGAGCGCGAGCAGGTGCCGAAGAAGCTTCTGAAGACG  960

Query  961   GTGATGATGAGGTTTCTGCCGGCCGCTGAGACGCTGCTGCAGATGATCGTGGCGCACCTG  1020
             |||||||||| ||| |||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  961   GTGATGATGAAGTTCCTGCCGGCTGCTGAGACGCTGCTGCAGATGATCGTGGCGCACCTG  1020

Query  1021  CCGTCGCCCAAGAGAGCGCAGGCGTACCGCGCGGAGATGTTGTACTCTGGTGAGGCGTCG  1080
             |||||||||||||  |||||||||||||||||||| |||||||||| |||||||||||||
Sbjct  1021  CCGTCGCCCAAGAAGGCGCAGGCGTACCGCGCGGAGATGCTGTACTCTGGCGAGGCGTCG  1080

Query  1081  CCGGAGGACAAGTACTTCATGGGTATCAAGAACTGCGACCCCGCTGCGCCGCTCATGCTG  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  CCGGAGGACAAGTACTTCATGGGTATCAAGAACTGCGACCCCGCTGCGCCGCTCATGCTG  1140

Query  1141  TACATCAGCAAGATGGTGCCGACGGCCGACCGCGGCCGCTTCTTCGCTTTTGGCCGCATC  1200
             |||||||||||||||||||||||||||||||||||||||||||||||| || ||||||||||
Sbjct  1141  TACATCAGCAAGATGGTGCCGACGGCCGACCGCGGCCGCTTCTTTGCCTTTGGCCGCATC  1200
```


Query 1201 TTCTCGGGTAAGGTGCGCAGCGGCCAGAAGGTGCGGATCATGGGCAACAACTACATCTAC 1260
Sbjct 1201 TTCTCCGGTAAGGTGCGCAGCGGCCAGAAGGTGCGCATCATGGGTAACAACTACGTCTAC 1260
Query 1261 GGCAAGAAGCAGGACCTGTACGATGACAAGCCTGTGCAGCGCTCTGTGCTGATGATGGGC 1320
Sbjct 1261 GGCAAGAAGCAGGACCTGTACGAGGACAAGCCTGTGCAGCGCTCCGTGCTGATGATGGGC 1320
Query 1321 CGCTACCAGGAGGCTGTGGAGGACATGCCGTGCGGTAACGTGGTGGGCCTTGTGGGCGTG 1380
Sbjct 1321 CGCTACCAGGAGGCCGTGGAGGACATGCCGTGCGGTAACGTGGTGGGCCTTGTGGGCGTG 1380
Query 1381 GACAAGTACATCGTGAAGTCTGCGACGATCACGGACGACGGCGAGAACCCGTACCCGCTG 1440
Sbjct 1381 GACAAGTACATTGTGAAGTCCGCGACGATCACGGACGACGGCGAGAGCCCGCACCCGCTG 1440
Query 1441 CGCGATATGAAGTACTCCGTGTCGCCTGTGGTGCGTGTGGCCGTGGAGGCGAAGAACCCG 1500
Sbjct 1441 CGCGACATGAAGTACTCTGTGTCGCCCGTCGTGCGTGTGGCCGTGGAGGCGAAGAACCCG 1500
Query 1501 TCCGACCTGCCGAAACTTGTGGAGGGCCTGAAGCGCCTTGCCAAGTCCGACCCGCTGGTG 1560
Sbjct 1501 TCCGACCTGCCGAAGCTTGTGGAGGGCCTGAAGCGCCTTGCCAAGTCCGACCCGCTGGTG 1560
Query 1561 GTGTGCAGCATTGAGGAGTCTGGCGAGCACATCGTTGCCGGCGCTGGTGAGCTGCATCTT 1620
Sbjct 1561 GTGTGCAGCATTGAGGAGTCTGGCGAGCACATTGTTGCCGGCGCTGGCGAGCTTCACCTT 1620
Query 1621 GAGATTTGCCTGAAGGACCTCCAGGAGGACTTCATGAACGGTGCGCCGCTGAAGATCTCC 1680
Sbjct 1621 GAGATTTGCCTGAAGGATCTCCAGGAGGACTTCATGAACGGCGCGCCGCTGAAGATCTCC 1680
Query 1681 GAGCCGGTGGTGTCGTTCCGCGAGACCGTGACGGACGTGTCGTCGCAGCAGTGTCTGTCG 1740
Sbjct 1681 GAGCCGGTGGTGTCGTTCCGCGAGACCGTGACGGACGTGTCGTCGCAGCAGTGCCTGTCG 1740
Query 1741 AAGTCTGCGAACAAGCACAACCGTCTGTTCTGCCGCGGTGCGCCGCTGACAGAGGAGCTT 1800
Sbjct 1741 AAGTCTGCGAACAAGCACAACCGTCTCTTCTGCCGCGGTGCGCCGCTGACAGAGGAGCTG 1800
Query 1801 GCGCTGGCGATGGAGGAGGGCACCGCTGGTCCCGAGGCCGATCCGAAGGTGCGCGCGCGC 1860
Sbjct 1801 GCGCTGGCGATGGAGGAGGGCACCGCTGGCCCCGAGGCGGATCCGAAGGTGCGCGCGCGC 1860
Query 1861 TTCCTTGCCGACAACTACGAGTGGGACGTGCAGGAGGCCCGCAAGATCTGGTGCTACGGC 1920
Sbjct 1861 TTCCTTGCCGACAACTACGAGTGGGATGTGCAGGAGGCCCGCAAGATCTGGTGCTACGGC 1920
Query 1921 CCGGACAACCGCGGCCCGAACGTGGTCGTGGATGTGACGAAGGGTGTCCAGAACATGGGT 1980
Sbjct 1921 CCGGACAACCGCGGCCCGAACGTGGTCGTGGATGTGACGAAGGGTGTCCAGAACATGGCT 1980
Query 1981 GAGATGAAGGACTCCTTTGTTGCGGCGTGGCAGTGGGCGACCCGCGAGGGTGTGCTCTGC 2040
Sbjct 1981 GAGATGAAGGACTCCTTTGTTGCGGCGTGGCAGTGGGCGACCCGCGAGGGTGTGCTCTGC 2040
Query 2041 GACGAGAACATGCGCGGTGTGCGCGTGAACGTGGAGGATGTGACGATGCACGCGGACGCC 2100
Sbjct 2041 GACGAGAACATGCGCGGCGTGCGCGTGAACGTGGAGGACGTGACGATGCACGCGGATGCC 2100
Query 2101 ATTCACCGTGGTGGCGGCCAGATCATCCCGACGGCGCGCCGTGTGTTCTACGCGTGCTGC 2160
Sbjct 2101 ATTCACCGTGGTGGCGGCCAGATCATCCCGACGGCGCGCCGCGTGTTCTACGCGTGCTGC 2160
Query 2161 CTGACGGCGTCGCCGCGCCTGATGGAGCCGATGTTCGTGGTGGATATCCAGACCGTGGAG 2220
Sbjct 2161 CTGACGGCGTCGCCGCGCCTGATGGAGCCGATGTTCGTGGTGGACATCCAGACCGTGGAG 2220
Query 2221 CACGCCATGGGCGGCATCTACGGTGTGCTGACCCGCCGCCGTGGTGTGATCATTGGCGAG 2280
Sbjct 2221 CACGCCATGGGCGGCATCTACGGTGTGCTGACCCGCCGCCGTGGCGTGATCATTGGCGAG 2280
Query 2281 GAGAACCGCCCGGGCACGCCCATCTACAACGTGCGCGCGTACCTGCCGGTTGCGGAGTCG 2340
Sbjct 2281 GAGAACCGCCCCGGCACGCCCATCTACAATGTGCGCGCGTACCTGCCGGTTGCGGAGTCG 2340
Query 2341 TTCGGCTTCACTGCCGACCTGCGCGCCGGAACTGGCGGCCAGGCCTTCCCGCAGTGCGTG 2400
Sbjct 2341 TTCGGCTTCACTGCCGACCTGCGCGCTGGAACTGGCGGCCAGGCCTTCCCGCAGTGCGTG 2400
Query 2401 TTCGACCACTGGCAGGAGTACCCCGGTGACCCGCTAGAGACCAAGTCGCTGGCCAACGCG 2460
Sbjct 2401 TTCGACCACTGGCAGGAGTACCCCGGCGACCCGCTGGAGCCGAAGTCGCTGGCCAACACG 2460
Query 2461 ACAACGCTTGCCATCCGCATGCGCAAGGGTCTGAAGCCGGAGATCCCCGGCCTGGACCAG 2520
Sbjct 2461 ACGACGCTTGCCATCCGCACGCGCAAGGGCCTGAAGCCGGATATCCCCGGCCTGGACCAG 2520
Query 2521 TTCATGGATAAATTGTAA 2538
Sbjct 2521 TTCATGGATAAGTTGTAA 2538

V – Alinhamento das sequências de aminoácidos da proteína NDKb de *L. braziliensis* e *L. infantum*

```
> LinJ.32.3110 | organism=Leishmania_infantum_JPCM5 | product=nucleoside
diphosphate kinase b | location=LinJ.32:1163891-1164346(-)
| length=151 | sequence_SO=chromosome | SO=protein_coding
Length=151

 Score =  293 bits (750),  Expect = 2e-101, Method: Compositional matrix adjust.
 Identities = 137/151 (91%), Positives = 146/151 (97%), Gaps = 0/151 (0%)

Query  1    MSSERTFIAIKPDGVQRGLVGEIISRFERKGFKLVALKMLQPTTEQAQGHYKDLASKPFF  60
            MSSERTFIA+KPDGVQRGLVGEII+RFERKG+KLVALK+LQPTTEQAQGHYKDL+SKPFF
Sbjct  1    MSSERTFIAVKPDGVQRGLVGEIIARFERKGYKLVALKILQPTTEQAQGHYKDLSSKPFF  60

Query  61   EGLVKYFSSGPIVCMVWEGKNVVKSGRVLLGATNPADSQPGTIRGDYAVDVGRNVCHGSD  120
              LVKYFSSGPIVCMVWEGKNVVKSGRVLLGATNPADSQPGTIRGD+AVDVGRNVCHGSD
Sbjct  61   PALVKYFSSGPIVCMVWEGKNVVKSGRVLLGATNPADSQPGTIRGDFAVDVGRNVCHGSD  120

Query  121  SVESAQREVAFWFKVEEIASWTSHSACQIYE  151
            SVESA+RE+AFWFK +EIASWTSHS  QIYE
Sbjct  121  SVESAEREIAFWFKADEIASWTSHSVSQIYE  151
```

VI – Alinhamento das sequências de aminoácidos da proteína EF2 de *L. braziliensis* e *L. infantum*

```
> LinJ.36.0190 | organism=Leishmania_infantum_JPCM5 | product=elongation
factor 2 (EF2-1) | location=LinJ.36:48610-51147(-)
| length=845 | sequence_SO=chromosome | SO=protein_coding
Length=845

 Score = 1730 bits (4480),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 829/845 (98%), Positives = 839/845 (99%), Gaps = 0/845 (0%)

Query  1    MVNFTVDQVRELMDFPDQIRNMSVIAHVDHGKSTLSDSLVGAAGIIKMEEAGDKRIMDTR  60
            MVNFTVDQVRELMD+PDQIRNMSVIAHVDHGKSTLSDSLVGAAGIIKMEEAGDKRIMDTR
Sbjct  1    MVNFTVDQVRELMDYPDQIRNMSVIAHVDHGKSTLSDSLVGAAGIIKMEEAGDKRIMDTR  60

Query  61   ADEIARGITIKSTAISMHYHVPKEMISSLDDDKRDFLINLIDSPGHVDFSSEVTAALRVT  120
            ADEIARGITIKSTAISMHYHVPKEMI  LDDDKRDFLINLIDSPGHVDFSSEVTAALRVT
Sbjct  61   ADEIARGITIKSTAISMHYHVPKEMIGDLDDDKRDFLINLIDSPGHVDFSSEVTAALRVT  120

Query  121  DGALVVVDCVEGVCVQTETVLRQALTERIRPVVFINKVDRAILELQLDPEEAYQGFVKTL  180
            DGALVVVDCVEGVCVQTETVLRQALTERIRPVVFINKVDRAILELQLDPEEAYQGFVKTL
Sbjct  121  DGALVVVDCVEGVCVQTETVLRQALTERIRPVVFINKVDRAILELQLDPEEAYQGFVKTL  180

Query  181  QNVNVVVATYNDPSMGDVQVSPEKGTVAIGSGLQAWAFSLTRFANMYASKFGVDELKMRE  240
            QNVNVVVATYNDPSMGDVQVSPEKGTVAIGSGLQAWAFSLTRFANMYA+KFGVDELKMRE
Sbjct  181  QNVNVVVATYNDPSMGDVQVSPEKGTVAIGSGLQAWAFSLTRFANMYAAKFGVDELKMRE  240

Query  241  RLWGDNFFDAKNKKWIKQETNADGERVRRAFCQFCLDPIYQIFDAVMNEKKDKVDKMLKS  300
            RLWGDNFFDAKNKKWIKQETNADGERVRRAFCQFCLDPIYQIFDAVMNEKKDKVDKMLKS
Sbjct  241  RLWGDNFFDAKNKKWIKQETNADGERVRRAFCQFCLDPIYQIFDAVMNEKKDKVDKMLKS  300

Query  301  LHVSLTAEEREQVPKKLLKTVMMRFLPAAETLLQMIVAHLPSPKRAQAYRAEMLYSGEAS  360
            LHV+LTAEEREQVPKKLLKTVMM+FLPAAETLLQMIVAHLPSPK+AQAYRAEMLYSGEAS
Sbjct  301  LHVTLTAEEREQVPKKLLKTVMMKFLPAAETLLQMIVAHLPSPKKAQAYRAEMLYSGEAS  360

Query  361  PEDKYFMGIKNCDPAAPLMLYISKMVPTADRGRFFAFGRIFSGKVRSGQKVRIMGNNYIY  420
            PEDKYFMGIKNCDPAAPLMLYISKMVPTADRGRFFAFGRIFSGKVRSGQKVRIMGNNY+Y
Sbjct  361  PEDKYFMGIKNCDPAAPLMLYISKMVPTADRGRFFAFGRIFSGKVRSGQKVRIMGNNYVY  420

Query  421  GKKQDLYDDKPVQRSVLMMGRYQEAVEDMPCGNVVGLVGVDKYIVKSATITDDGENPYPL  480
            GKKQDLY+DKPVQRSVLMMGRYQEAVEDMPCGNVVGLVGVDKYIVKSATITDDGE+P+PL
Sbjct  421  GKKQDLYEDKPVQRSVLMMGRYQEAVEDMPCGNVVGLVGVDKYIVKSATITDDGESPHPL  480

Query  481  RDMKYSVSPVVRVAVEAKNPSDLPKLVEGLKRLAKSDPLVVCSIEESGEHIVAGAGELHL  540
            RDMKYSVSPVVRVAVEAKNPSDLPKLVEGLKRLAKSDPLVVCSIEESGEHIVAGAGELHL
Sbjct  481  RDMKYSVSPVVRVAVEAKNPSDLPKLVEGLKRLAKSDPLVVCSIEESGEHIVAGAGELHL  540

Query  541  EICLKDLQEDFMNGAPLKISEPVVSFRETVTDVSSQQCLSKSANKHNRLFCRGAPLTEEL  600
            EICLKDLQEDFMNGAPLKISEPVVSFRETVTDVSSQQCLSKSANKHNRLFCRGAPLTEEL
Sbjct  541  EICLKDLQEDFMNGAPLKISEPVVSFRETVTDVSSQQCLSKSANKHNRLFCRGAPLTEEL  600

Query  601  ALAMEEGTAGPEADPKVRARFLADNYEWDVQEARKIWCYGPDNRGPNVVVDVTKGVQNMG  660
            ALAMEEGTAGPEADPKVRARFLADNYEWDVQEARKIWCYGPDNRGPNVVVDVTKGVQNM
Sbjct  601  ALAMEEGTAGPEADPKVRARFLADNYEWDVQEARKIWCYGPDNRGPNVVVDVTKGVQNMA  660

Query  661  EMKDSFVAAWQWATREGVLCDENMRGVRVNVEDVTMHADAIHRGGGQIIPTARRVFYACC  720
            EMKDSFVAAWQWATREGVLCDENMRGVRVNVEDVTMHADAIHRGGGQIIPTARRVFYACC
Sbjct  661  EMKDSFVAAWQWATREGVLCDENMRGVRVNVEDVTMHADAIHRGGGQIIPTARRVFYACC  720

Query  721  LTASPRLMEPMFVVDIQTVEHAMGGIYGVLTRRRGVIIGEENRPGTPIYNVRAYLPVAES  780
            LTASPRLMEPMFVVDIQTVEHAMGGIYGVLTRRRGVIIGEENRPGTPIYNVRAYLPVAES
Sbjct  721  LTASPRLMEPMFVVDIQTVEHAMGGIYGVLTRRRGVIIGEENRPGTPIYNVRAYLPVAES  780

Query  781  FGFTADLRAGTGGQAFPQCVFDHWQEYPGDPLETKSLANATTLAIRMRKGLKPEIPGLDQ  840
            FGFTADLRAGTGGQAFPQCVFDHWQEYPGDPLE KSLAN TTLAIR RKGLKP+IPGLDQ
Sbjct  781  FGFTADLRAGTGGQAFPQCVFDHWQEYPGDPLEPKSLANTTTLAIRTRKGLKPDIPGLDQ  840

Query  841  FMDKL  845
            FMDKL
Sbjct  841  FMDKL  845
```

VII – Alinhamento das sequências de aminoácidos da proteína NDKb de mamífero e *L. braziliensis*

```
> LbrM.32.3210 | organism=Leishmania_braziliensis_MHOM/BR/75/M2904
| product=nucleoside diphosphate kinase b | location=LbrM.32:1182666-1183121(-)
| length=151 | sequence_SO=chromosome
| SO=protein_coding
Length=151

 Score =  213 bits (542),  Expect = 8e-70, Method: Compositional matrix adjust.
 Identities = 97/148 (66%), Positives = 121/148 (82%), Gaps = 0/148 (0%)

Query  5    ERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVAMKFLRASEEHLKQHYIDLKDRPFFPGL  64
            ERTFIAIKPDGVQRGLVGEII RFE+KGF+LVA+K L+ + E  + HY DL  +PFF GL
Sbjct  4    ERTFIAIKPDGVQRGLVGEIISRFERKGFKLVALKMLQPTTEQAQGHYKDLASKPFFEGL  63

Query  65   VKYMNSGPVVAMVWEGLNVVKTGRVMLGETNPADSKPGTIRGDFCIQVGRNIIHGSDSVK  124
            VKY +SGP+V MVWEG NVVK+GRV+LG TNPADS+PGTIRGD+ + VGRN+ HGSDSV+
Sbjct  64   VKYFSSGPIVCMVWEGKNVVKSGRVLLGATNPADSQPGTIRGDYAVDVGRNVCHGSDSVE  123

Query  125  SAEKEISLWFKPEELVDYKSCAHDWVYE  152
            SA++E++ WFK EE+  + S +   +YE
Sbjct  124  SAQREVAFWFKVEEIASWTSHSACQIYE  151
```

VIII – Alinhamento das sequências de aminoácidos da proteína NDKb de mamífero e *L. infantum*

```
> LinJ.32.3110 | organism=Leishmania_infantum_JPCM5 | product=nucleoside
diphosphate kinase b | location=LinJ.32:1163891-1164346(-)
| length=151 | sequence_SO=chromosome | SO=protein_coding
Length=151

 Score =  216 bits (549),  Expect = 8e-71, Method: Compositional matrix adjust.
 Identities = 97/148 (66%), Positives = 121/148 (82%), Gaps = 0/148 (0%)

Query  5    ERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVAMKFLRASEEHLKQHYIDLKDRPFFPGL  64
            ERTFIA+KPDGVQRGLVGEII RFE+KG++LVA+K L+ + E  + HY DL  +PFFP L
Sbjct  4    ERTFIAVKPDGVQRGLVGEIIARFERKGYKLVALKILQPTTEQAQGHYKDLSSKPFFPAL  63

Query  65   VKYMNSGPVVAMVWEGLNVVKTGRVMLGETNPADSKPGTIRGDFCIQVGRNIIHGSDSVK  124
            VKY +SGP+V MVWEG NVVK+GRV+LG TNPADS+PGTIRGDF + VGRN+ HGSDSV+
Sbjct  64   VKYFSSGPIVCMVWEGKNVVKSGRVLLGATNPADSQPGTIRGDFAVDVGRNVCHGSDSVE  123

Query  125  SAEKEISLWFKPEELVDYKSCAHDWVYE  152
            SAE+EI+ WFK +E+  + S +   +YE
Sbjct  124  SAEREIAFWFKADEIASWTSHSVSQIYE  151
```

IX – Alinhamento das sequências de aminoácidos da proteína EF2 de mamífero e *L. braziliensis*

```
> LbrM.35.0270 | organism=Leishmania_braziliensis_MHOM/BR/75/M2904
| product=elongation factor 2 | location=LbrM.35:84057-86594(-)
| length=845 | sequence_SO=chromosome | SO=protein_coding
Length=845

 Score = 1083 bits (2800),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 530/863 (61%), Positives = 656/863 (76%), Gaps = 23/863 (3%)

Query  1    MVNFTVDQIRAIMDKKANIRNMSVIAHVDHGKSTLTDSLVCKAGIIASARAGETRFTDTR  60
            MVNFTVDQ+R +MD    IRNMSVIAHVDHGKSTL+DSLV  AGII    AG+ R  DTR
Sbjct  1    MVNFTVDQVRELMDFPDQIRNMSVIAHVDHGKSTLSDSLVGAAGIIKMEEAGDKRIMDTR  60

Query  61   KDEQERCITIKSTAISLFYELSENDLNFIKQSKDGAGFLINLIDSPGHVDFSSEVTAALR  120
             DE  R ITIKSTAIS+ Y + +  ++ +    D   FLINLIDSPGHVDFSSEVTAALR
Sbjct  61   ADEIARGITIKSTAISMHYHVPKEMISSL--DDDKRDFLINLIDSPGHVDFSSEVTAALR  118

Query  121  VTDGALVVVDCVSGVCVQTETVLRQAIAERIKPVLMMNKMDRALLELQLEPEELYQTFQR  180
            VTDGALVVVDCV GVCVQTETVLRQA+ ERI+PV+ +NK+DRA+LELQL+PEE YQ F +
Sbjct  119  VTDGALVVVDCVEGVCVQTETVLRQALTERIRPVVFINKVDRAILELQLDPEEAYQGFVK  178

Query  181  IVENVNVIISTYGEGESGPMGNIMIDPVLGTVGFGSGLHGWAFTLKQFAEMYVAKFAAKG  240
             ++NVNV+++TY +     MG++ + P  GTV  GSGL  WAF+L +FA MY +KF   G
Sbjct  179  TLQNVNVVVATYNDPS---MGDVQVSPEKGTVAIGSGLQAWAFSLTRFANMYASKF---G  232

Query  241  EGQLGPAERAKKVEDMMKKLWGDRYFDPANGKFSKSATSPEGKKLPRTFCQLILDPIFKV  300
              +L           M ++LWGD +FD  N K+ K  T+ +G+++ R FCQ  LDPI+++
Sbjct  233  VDEL----------KMRERLWGDNFFDAKNKKWIKQETNADGERVRRAFCQFCLDPIYQI  282

Query  301  FDAIMNFKKEETAKLIEKLDIKLDSEDKDKEGKPLLKAVMRRWLPAGDALLQMITIHLPS  360
            FDA+MN KK++  K++ L + L +E+++  K LLK VM R+LPA + LLQMI  HLPS
Sbjct  283  FDAVMNEKKDKVDKMLKSLHVSLTAEEREQVPKKLLKTVMMRFLPAAETLLQMIVAHLPS  342

Query  361  PVTAQKYRCELLYEG--PPDDEAAMGIKSCDPKGPLMMYISKMVPTSDKGRFYAFGRVFS  418
            P  AQ YR E+LY G   P+D+  MGIK+CDP  PLM+YISKMVPT+D+GRF+AFGR+FS
Sbjct  343  PKRAQAYRAEMLYSGEASPEDKYFMGIKNCDPAAPLMLYISKMVPTADRGRFFAFGRIFS  402

Query  419  GLVSTGLKVRIMGPNYTPGKKEDLYL-KPIQRTILMMGRYVEPIEDVPCGNIVGLVGVDQ  477
            G V +G KVRIMG NY  GKK+DLY  KP+QR++LMMGRY E +ED+PCGN+VGLVGVD+
Sbjct  403  GKVRSGQKVRIMGNNYIYGKKQDLYDDKPVQRSVLMMGRYQEAVEDMPCGNVVGLVGVDK  462

Query  478  FLVKTGTITT-FEHAHNMRVMKFSVSPVVRVAVEAKNPADLPKLVEGLKRLAKSDPMVQC  536
            ++VK+ TIT   E+ + +R MK+SVSPVVRVAVEAKNP+DLPKLVEGLKRLAKSDP+V C
Sbjct  463  YIVKSATITDDGENPYPLRDMKYSVSPVVRVAVEAKNPSDLPKLVEGLKRLAKSDPLVVC  522

Query  537  IIEESGEHIIAGAGELHLEICLKDLEEDHAC-IPIKKSDPVVSYRETVSEESNVLCLSKS  595
             IEESGEHI+AGAGELHLEICLKDL+ED     P+K S+PVVS+RETV++ S+  CLSKS
Sbjct  523  SIEESGEHIVAGAGELHLEICLKDLQEDFMNGAPLKISEPVVSFRETVTDVSSQQCLSKS  582

Query  596  PNKHNRLYMKARPFPDGLAEDIDKGEVSARQELKQRARYLAEKYEWDVAEARKIWCFGPD  655
             NKHNRL+ +  P  + LA  +++G      + K RAR+LA+ YEWDV EARKIWC+GPD
Sbjct  583  ANKHNRLFCRGAPLTEELALAMEEGTAGPEADPKVRARFLADNYEWDVQEARKIWCYGPD  642

Query  656  GTGPNILTDITKGVQYLNEIKDSVVAGFQWATKEGALCEENMRGVRFDVHDVTLHADAIH  715
              GPN++ D+TKGVQ + E+KDS VA +QWAT+EG LC+ENMRGVR +V DVT+HADAIH
Sbjct  643  NRGPNVVVDVTKGVQNMGEMKDSFVAAWQWATREGVLCDENMRGVRVNVEDVTMHADAIH  702

Query  716  RGGGQIIPTARRCLYASVLTAQPRLMEPIYLVEIQCPEQVVGGIYGVLNRKRGHVFEESQ  775
            RGGGQIIPTARR  YA  LTA PRLMEP+++V+IQ  E  +GGIYGVL R+RG +  E
Sbjct  703  RGGGQIIPTARRVFYACCLTASPRLMEPMFVVDIQTVEHAMGGIYGVLTRRRGVIIGEEN  762

Query  776  VAGTPMFVVKAYLPVNESFGFTADLRSNTGGQAFPQCVFDHWQILPGDPFDNSSRPSQVV  835
              GTP++ V+AYLPV ESFGFTADLR+ TGGQAFPQCVFDHWQ  PGDP +  S  +
Sbjct  763  RPGTPIYNVRAYLPVAESFGFTADLRAGTGGQAFPQCVFDHWQEYPGDPLETKSLANATT  822

Query  836  AETRKRKGLKEGIPALDNFLDKL  858
               R RKGLK  IP LD F+DKL
Sbjct  823  LAIRMRKGLKPEIPGLDQFMDKL  845
```

X – Alinhamento das sequências de aminoácidos da proteína EF2 de mamífero e *L. infantum*

```
> LinJ.36.0190 | organism=Leishmania_infantum_JPCM5 | product=elongation
factor 2 (EF2-1) | location=LinJ.36:48610-51147(-)
| length=845 | sequence_SO=chromosome | SO=protein_coding
Length=845

 Score = 1083 bits (2801),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 531/863 (62%), Positives = 654/863 (76%), Gaps = 23/863 (3%)

Query  1    MVNFTVDQIRAIMDKKANIRNMSVIAHVDHGKSTLTDSLVCKAGIIASARAGETRFTDTR  60
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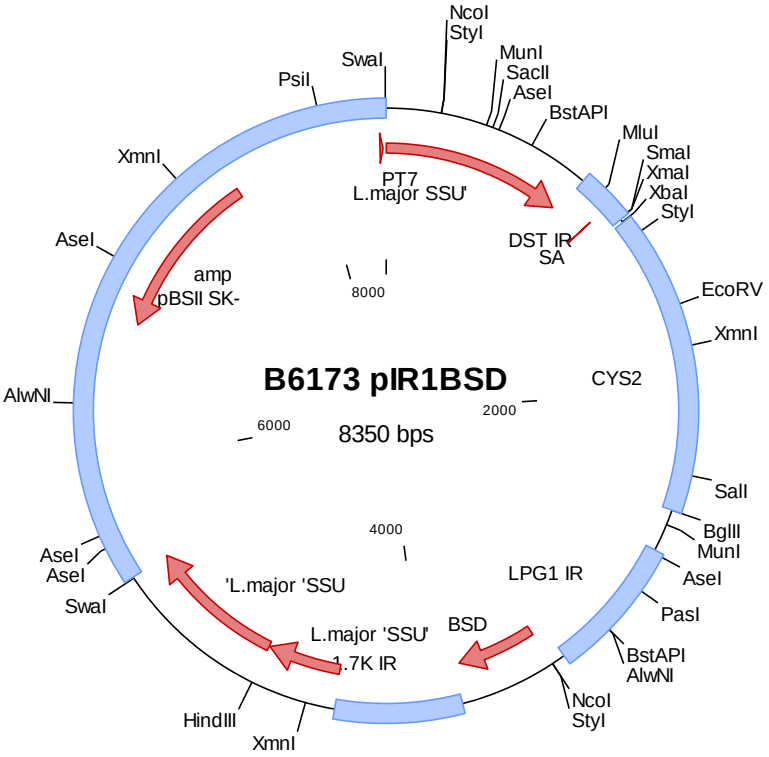
XI – Mapa do plasmídeo pIR1BSD utilizado nos ensaios de transfecção



Plasmídeo pIR1-BSD. As regiões em azul indicam as regiões intergênicas: CYS2, LPG1, 1.7K e pBSII SK. Indicado com as setas vermelhas estão: gene que confere resistência à blasticidina (BSD), as regiões da subunidade pequena do RNA ribossomal de *L. major* (*L. major* SSU) onde o plasmídeo se integrará no genoma de *Leishmania* spp. após a transfecção, e o gene que confere resistência à ampicilina (amp). Este plasmídeo foi gentilmente cedido pelo Dr. Stephen Beverley (Washington University – EUA).

XII – Produções científicas realizadas durante o Doutorado

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Molecular characterization of the MRPA transporter and antimony uptake in four New World *Leishmania* spp. susceptible and resistant to antimony ☆

Douglas S. Moreira [a], Rubens L. Monte Neto [b,c], Juvana M. Andrade [a], Ana Maria M. Santi [a], Priscila G. Reis [b], Frédéric Frézard [b], Silvane M.F. Murta [a,*]

[a] Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou – CPqRR/FIOCRUZ, Belo Horizonte 30190-002, Minas Gerais, Brazil
[b] Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte 31270-901, Minas Gerais, Brazil
[c] Centre de Recherche en Infectiologie, CHUL, Québec G1V 4G2, QC, Canada

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ABSTRACT

ATP-binding cassette (ABC) transporters have been associated with drug resistance in various diseases. The *MRPA* gene, a transporter of ABCC subfamily, is involved in the resistance by sequestering metal-thiol conjugates in intracellular vesicles of *Leishmania* parasite. In this study, we performed the molecular characterization of the MRPA transporter, analysis of P-glycoprotein (Pgp) and aquaglyceroporin-1 (AQP1) expression, and determination of antimony level in antimony-susceptible and -resistant lines of *L. (V.) guyanensis*, *L. (L.) amazonensis*, *L. (V.) braziliensis* and *L. (L.) infantum*. PFGE analysis revealed an association of chromosomal amplification of *MRPA* gene with the drug resistance phenotype in all SbIII-resistant *Leishmania* lines analyzed. Levels of mRNA from *MRPA* gene determined by real-time quantitative RT-PCR showed an increased expression of two fold in SbIII-resistant lines of *Leishmania guyanensis*, *Leishmania amazonensis* and *Leishmania braziliensis*. Western blot analysis revealed that Pgp is increased in the SbIII-resistant *L. guyanensis* and *L. amazonensis* lines. The intracellular level of antimony quantified by graphite furnace atomic absorption spectrometry showed a reduction in the accumulation of this element in SbIII-resistant *L. guyanensis*, *L. amazonensis* and *L. braziliensis* lines when compared to their susceptible counterparts. Interestingly, a down-regulation of AQP1 protein was observed in the SbIII-resistant *L. guyanensis* and *L. amazonensis* lines, contributing for decreasing of SbIII entry in these lines. In addition, efflux experiments revealed that the rates of SbIII efflux are higher in the SbIII-resistant lines of *L. guyanensis* and *L. braziliensis*, that may explain also the low SbIII concentration within of these parasites. The BSO, an inhibitor of γ-glutamylcysteine synthetase enzyme, reversed the SbIII-resistance phenotype of *L. braziliensis* and caused an increasing in the Sb intracellular level in the LbSbR line. Our data indicate that the mechanisms of antimony-resistance are different among species of *Leishmania* analyzed in this study.

1. Introduction

Leishmaniasis is a complex of diseases caused by different species of protozoan parasites belonging to the genus *Leishmania*. This neglected tropical disease comprises clinical manifestations that range from self-healing cutaneous (CL) and mucocutaneous (MCL) skin lesions to a visceral (VL) form, which is lethal if untreated (Ashutosh et al., 2007). In the New World, *L. (Leishmania) infantum* (syn. *L. (L.) chagasi*) (Kuhls et al., 2007) is the causative agent of VL, whereas *L. (L.) amazonensis* and *L. (Viannia) guyanensis* cause CL, and *L. (V.) braziliensis* causes CL and MCL (Marzochi and Marzochi, 1994; Murray et al., 2005; Shaw, 2006). The disease is endemic in 98 countries, especially in northern Africa, Asia, the Mediterranean and Latin America, with more than 350 million people at risk (Ashford et al., 1992; Alvar et al., 2012). There are an estimated 12 million people infected worldwide (World Health Organization, 2012). The estimated incidence of leishmaniasis is approximately 0.2–0.4 million VL cases and 0.7–1.2 million CL cases per year. More than 90% of global VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and

Abbreviations: SbIII, potassium antimonyl tartrate; WTS, Wild-type susceptible; SbR, SbIII-resistant; Lg, *L. (V.) guyanensis*; Lb, *L. (V.) braziliensis*; La, *L. (L.) amazonensis*; Li, *L. (L.) infantum*; MRPA, multidrug-resistance protein A; Pgp, phosphoglycoprotein; AQP1, aquaglyceroporin-1.

* Corresponding author. Address: Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas 'René Rachou', FIOCRUZ, Av. Augusto de Lima 1715, Caixa Postal 1743, CEP 30190-002 Belo Horizonte, MG, Brazil. Tel.: +55 31 3349 7780; fax: +55 31 32953115.
E-mail address: silvane@cpqrr.fiocruz.br (S.M.F. Murta).

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Brazil. Whereas Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru account for 70–75% of global estimated CL incidence (Alvar et al., 2012).

The control of leishmaniasis relies mainly on chemotherapy. The first line choice of treatment against all forms of the disease is based on the administration of pentavalent antimony-containing compounds, such as sodium stibogluconate (SSG) (Pentostam®) and N-methyl-glucamine (Glucantime®). The mechanisms of action of antimony are not fully elucidated. It is known that SbV acts on the amastigote form, inhibiting enzyme activity and the oxidative pathway of fatty acids (Herwaldt, 1999). It is generally agreed that trivalent antimony (SbIII) is the active form of the drug against *Leishmania* amastigote and promastigote forms (Frézard et al., 2009). Murray and Nathan (1988) demonstrated that macrophage activation has a significant effect on intracellular parasite killing. It has been reported that SSG induces the macrophage to produce leishmanicidal molecules like nitric oxide (NO) and reactive oxygen species (ROS) by activation of signalling pathways, leading to the elimination of intracellular *Leishmania donovani* amastigotes (Basu et al., 2006). In an animal infection model, the mode of action of SbV is dependent on a number of factors including T cell subsets and cytokines (reviewed by Murray, (2001)). Furthermore, SbV was found to be a potent inhibitor of protein tyrosine phosphatases, leading to an increase in cytokine responses (Pathak and Yi, 2001). Thus, these results suggest that SSG may kill the parasites by both direct and indirect mechanisms. Studies suggest that SbIII causes disturbances in the thiol redox potential of the parasite by inducing the efflux of intracellular thiols and by inhibiting trypanothione reductase, leading the cell to death by oxidative stress (Wyllie et al., 2004; Moreira et al., 2011). It has been shown that antimony kills the parasite by a process involving DNA fragmentation and externalization of phosphatidylserine on the outer surface of membrane (Sereno et al., 2001; Lee et al., 2002; Sudhandiran and Shaha, 2003).

Drug resistance is one of the major clinical problems for the treatment of various diseases ranging from bacteria and parasite infections to cancer. Treatment failure with pentavalent antimonials has been reported recently in several countries. In India, over 60% of patients with VL do not respond to treatment with pentavalent antimony drugs, due to acquired resistance (Sundar et al., 2000). The mechanisms by which species of *Leishmania* acquire resistance to antimonials have been subject of intensive research for several decades. It has been described that resistance is an interplay between uptake, efflux and sequestration of active molecules (reviewed by Croft et al., 2006). A down-regulation of aquaglyceroporin-1 (AQP1) is correlated with lower SbIII uptake, decreasing the drug concentration within the cell (Marquis et al., 2005). On the other hand, amplification of DNA segments has been observed in several *Leishmania* species selected for drug resistance (Beverley, 1991; Ouellette and Borst, 1991).

ATP-binding cassette (ABC) transporters have been associated with drug resistance in various diseases. These transporters comprise an ancient superfamily of evolutionarily conserved proteins spanning from bacteria to humans (Dassa and Bouige, 2001). They are integral membrane proteins involved in the energy-dependent transport of a variety of molecules across biological membranes, including amino acids, sugars, peptides, lipids, ions and chemotherapeutic drugs (Higgins, 1992). In mammals, these transporters are associated with chemoresistance mainly through overexpression of the multidrug-resistance (MDR) proteins (Borst et al., 2000). In *Leishmania*, the first ABC protein identified was MRPA (PgpA) (Ouellette et al., 1990) which is a member of the ABCC subfamily able to confer resistance to antimonials by sequestering metal-thiol conjugates into an intracellular vesicle (Légaré et al., 2001).

Different mechanisms of drug resistance have been identified in Old Word *Leishmania* species (reviewed by Croft et al., 2006). Although there is considerable evidence showing variability in the response to antimony chemotherapy in New World pathogenic *Leishmania* species (Moreira et al., 1998; Romero et al., 2001), the mechanisms of drug resistance in these species have not been extensively analyzed. Therefore, understanding the biological diversity and responses to chemotherapy of different New World *Leishmania* species is necessary to ultimately overcome current limitations in anti-parasitic drug treatment. Thus, in this study we performed the molecular characterization of the MRPA transporter and determination of antimony level in antimony-susceptible and -resistant lines, which were experimentally induced, in four New World *Leishmania* species: *Leishmania guyanensis*, *Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania infantum*. Promastigote forms were characterized for: chromosomal location, analysis of amplification and mRNA levels of the *MRPA* gene; Pgp and APQ1 protein expression; measurement of intracellular antimony level, efflux rates of SbIII and susceptibility of *L. braziliensis* lines to BSO, an inhibitor of γ-glutamylcysteine synthetase (GCS) enzyme.

2. Materials and methods

2.1. Leishmania strains

In this study, we used promastigote forms of four different New World *Leishmania* species: *L. guyanensis*, *L. amazonensis*, *L. braziliensis* and *L. infantum* (Table 1). These lines were selected *in vitro* to trivalent antimony (SbIII) by step-wise drug pressure and the resistance index varied from 4 to 20-fold higher than of their wild-type counterparts (Liarte and Murta, 2010). This previous study showed that the SbIII-resistant lines of *L. amazonensis*, *L. braziliensis* and *L. infantum* have cross-resistance to paromomycin. Parasites were grown at 26 °C in M199 medium (Liarte and Murta, 2010).

2.2. Pulsed field gel electrophoresis (PFGE)

Chromosomal DNA from *Leishmania* lines (2.0×10^9 cells/mL) was prepared in low-melting temperature agarose plugs as described by Smith et al. (1988). The agarose blocks containing intact *Leishmania* chromosomes were separated by PFGE in 1% agarose gels in 0.5x TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.3) using the Gene Navigator TM System (Amersham Pharmacia, Buckinghamshire, UK). The running buffer (0.5x TBE) was maintained at 9 °C throughout the electrophoresis. *Saccharomyces cerevisiae* chromosomes were used as size markers (Bio-Rad Inc., Hercules, CA, USA). Electrophoresis conditions were standardized to allow the separation of the greatest number of parasite chromosomes in a single gel, as follows: 90 s for 18 h, 200 s for 18 h, 400 s for 22 h and 600 s for 7 h at 90 V. A range from 365 to 2200 kb was used for wide separation of *Leishmania* chromosomes. After electrophoresis, gels were stained with ethidium bromide (10 μg/mL) and bands were then transferred onto nylon membrane (Sambrook et al., 1989). The *MRPA* gene was identified by incubation of the membrane with the ^{32}P-labelled *MRPA* gene probe. For this probe, was used a 452 bp MRPA fragment (*LbrM23_V2.0280*) amplified with the primers: forward 5'-TCGTGATTATTCCGTGCGCGTT-3' and reverse 5'-ACGCTCCACGCTGTTCATGTTT-3'.

2.3. Southern blot

Genomic DNA was isolated from the antimony-susceptible and -resistant *Leishmania* species according to the protocol described by Sambrook et al. (1989). Approximately 10 μg total DNA of

Table 1
Leishmania species used in this study*.

Species	Strains	IC_{50} (μM)		Resistance index
		WTS	SbR	
L. (V.) guyanensis	IUMB/BR/85/M9945	0.09 ± 0.04	1.64 ± 0.14	19
L. (L.) amazonensis	IFLA/BR/67/PH8	0.28 ± 0.15	1.71 ± 0.11	6
L. (V.) braziliensis	MHOM/BR/75/M2904	0.15 ± 0.15	3.04 ± 0.13	20
L. (L.) infantum	MHOM/BR/74/PP75	0.33 ± 0.09	1.40 ± 0.04	4

IC_{50} is the concentration (μM) of SbIII that decreases the rate of cell growth by 50%.The values represent the means ± CI (confidence interval) of three independent experiments in triplicate.
* Reference: Liarte and Murta (2010).

Leishmania lines were digested with the restriction enzyme *Bam*HI (Invitrogen, Carlsbad, CA, USA). The fragments were separated by electrophoresis on a 1% agarose gel and transferred to nylon membrane (Sambrook et al., 1989). The blots were hybridized with [α-^{32}P]dCTP labeled *MRPA* gene probe as described above. *Leishmania DNA polymerase* gene (*LbrM.16.1600*) was used as quantitative control. A fragment of 483 bp from this gene, after amplification using primers: forward 5'-GAAGACAGAGAAGGATGCGA-3' and reverse 5'-GAGAGCGGGCACCAATCAC-3', was used as probe. The band intensities were analyzed using the software CP ATLAS 2.0 (http://lazarsoftware.com/download.html).

2.4. Quantitative real-time RT-PCR

Reverse transcription reactions for first strand complementary DNA (cDNA) synthesis were carried out as described below. Each reaction contained 2 μg total RNA, 0.5 μg oligo d(T), 1x first strand buffer, 10 mM DTT, 0.5 mM dNTP, 40 U RNasin and 200 U Superscript II reverse transcriptase in a final volume of 20 μL. All the reagents used were obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). All reactions were allowed to proceed for 1 h at 42 °C before being stopped by incubation at 70 °C for 20 min. The obtained cDNA was then diluted 15x in water and 5 μL of the reaction product was amplified by real-time PCR using specific primers. Amplification reaction was carried out using the 7000 System SDS Software (PE Applied Biosystems, Foster City, CA, USA). The primers, *MRPA* forward: 5'-AAGTGGGCAGCGACTCAAA-3' and *MRPA* reverse: 5'-CCAGTTCAGCGTCTCCGTT-3', were the same described by Torres et al. (2010) and Adaui et al. (2011). The *DNA polymerase* constitutive gene from *L. braziliensis* (*LbrM.16.1600*) was used to normalize the amount of sample analyzed. A fragment of 69 bp of the *DNA polymerase* gene was amplified with the primers forward: 5'-CGAGGGCAAGACATAC-3' and reverse: 5'-GAGAGCGGGCACCAATCAC-3'. Both pair of primers *MRPA* and *DNA polymerase* amplified fragments of 81 and 69 bp respectively, in all four *Leishmania* species analyzed (data not shown). PCR was carried out in a final volume of 20 μL of reaction mixture containing 10 pmol of forward and reverse primers, 1x SYBR GREEN reaction mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and 5 μL of template DNA. The PCR conditions were as follows: an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Standard curves were prepared for each run using known quantities of TOPO PCR 2.1 plasmids (Invitrogen, Life Technologies, Carlsbad, CA, USA) containing the *MRPA* and *DNA polymerase* genes. Estimates of DNA levels were obtained using the Sequence Detection System data analysis software. Values were normalized to those obtained for *DNA polymerase* for each sample.

2.5. Western blot analysis

Total proteins and membrane protein fractions from different *Leishmania* lines were extracted according to the protocols described by Gamarro et al. (1994) and Grogl et al. (1991), respectively. Protein extracts were separated by electrophoresis on a 12% SDS polyacrylamide gel and electrotransferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The blots were blocked by incubation with 5% instant non-fat dry milk in PBS supplemented with 0.05% Tween 20 (PBS-T) for 1 h. The blots were then washed twice in PBS-T and incubated for 16 h at 4 °C in the presence of antibodies: monoclonal antibody C219 (1:100) (Abcam, Cambridge, UK) or rabbit polyclonal antibody anti-AQP1 (1:5) (kindly provided by Andrade et al. – in preparation). The blots were washed and incubated with anti-IgG mouse HRP-conjugate (1:2,000) (GE Healthcare) or HRP-conjugated anti-rabbit IgG (1:5,000) (GE Healthcare) for 1 h at room temperature. After, the blots were exposed to Amersham ECL Plus detection agent according to the manufacturer's instructions and exposed to an X-ray film (Amersham, Buckinghamshire, UK). The results were normalized using the anti-α-tubulin monoclonal antibody (Sigma, St. Louis, USA). The intensity of the bands was analyzed using the software CP ATLAS 2.0.

2.6. Antimony transport assays

2.6.1. Uptake assay

Before performing the uptake and efflux assays, the SbIII-resistant lines are maintained at least two passages in M199 medium in the absence of SbIII, in order to eliminate the residual drug. The antimony uptake assay was based on two protocols previously described (Roberts and Rainey, 1993; Wang et al., 2003). Log phase *Leishmania* promastigotes were washed twice in a HEPES/Glucose (HG) buffer (20 mM HEPES, 0.15 M NaCl, 10 mM glucose, pH 7.2) and resuspended in this buffer at a density of 1.0×10^8 cells/mL. The volume of 1 mL of this parasite suspension was aliquoted into tubes in quadruplicate: tubes containing only parasites (blank) and tubes with parasites in the presence of 540 μM SbIII (potassium antimonyl tartrate) (Sigma, Saint Louis, MO, USA). Cells were then incubated at 26 °C for 1 h under agitation. Subsequently, the cells were centrifuged at 1816g for 5 min at 4 °C and washed three times with HG buffer. Pellets were then resuspended with 100 μL HG buffer. One aliquot of 10 μL of each tube was used for normalization (parasite quantification) and the remaining volume (90 μL) was submitted to digestion with nitric acid (65%). Antimony concentration was quantified by graphite furnace atomic absorption spectrometry (Perkin Elmer AAnalyst 600). Each uptake assay was performed three times and the signal from blanks was used for background subtraction.

In addition, the antimony-susceptible and -resistant lines of the species *L. guyanensis* and *L. braziliensis* were evaluated for the kinetics of incorporation of Sb. The parasites were incubated with 540 μM SbIII and, at different time intervals (0, 0.5, 1, 2 and 3 h), 1 mL aliquots of the parasite suspension were taken and they were submitted to the protocol described above. This assay was also performed to identify the time points at which the WTS and SbR lines exhibit the same intracellular concentration of Sb, to be explored in the efflux protocol.

2.6.2. Efflux assay

Log phase antimony-susceptible and -resistant *L. guyanensis* and *L. braziliensis* cells were washed twice with HG buffer and resuspended in this buffer at a density of 1.0×10^8 cells/mL. Aliquots of 1 mL containing only parasites (blank) were taken and the remaining cells were incubated with 540 μM SbIII for each 1 mL (potassium antimonyl tartrate) (Sigma, Saint Louis, MO, USA) at 26 °C under agitation. The WTS lines of *L. guyanensis* and *L. braziliensis* were incubated during 2 min and 30 min, respectively. Both SbR lines of these species were incubated for 70 min. After incubation, the cells were centrifuged at 890g for 5 min at 4 °C and washed two times with HG buffer. The pellets were then resuspended with HG buffer at the original density and the cells were incubated at 26 °C under agitation. Aliquots of 1 mL were taken from the parasite suspension at the times 0, 1 and 2 h. Subsequently, these aliquots and the blanks were centrifuged at 890g for 5 min at 4 °C and washed three times with HG buffer. After the last centrifugation, the pellets were resuspended with 100 μL HG buffer. One aliquot of 10 μL of each tube was used for normalization (parasite quantification) and the remaining volume (90 μL) was submitted to digestion with nitric acid (65%). Antimony concentration was quantified by graphite furnace atomic absorption spectrometry (Perkin Elmer AAnalyst 600). Each efflux assay was performed three times in triplicate and the SbIII dosage from blanks was used for background subtraction.

2.6.3. Test of susceptibility to BSO and SbIII uptake in L. braziliensis lines pre-treated with this inhibitor

Approximately 2.0×10^6 cells/mL of *L. braziliensis* were grown in M199 medium containing various concentrations (2.5–30 mg/mL) of buthionine-sulphoximine (BSO) (Sigma) during 48 h at 26 °C. BSO is an inhibitor of γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme in thiol biosynthesis (Griffith and Meister, 1979). The drug concentration that decreases cell growth by 50% (IC_{50}) was determined by counting the parasites in the presence and absence of the BSO. After pre-treatment with BSO, the uptake of Sb was investigated in both susceptible and resistant *L. braziliensis* lines. These parasites were first grown in M199 medium for 48 h in the presence BSO at their respective IC_{50}. Subsequently, the cells were washed twice with HG buffer, resuspended in this buffer at a density of 1.0×10^8 cells/mL and submitted to the uptake assay.

2.7. Statistical analysis

Data were analyzed by Student's *t* test performed using the software GraphPad Prism 5.0. A p value of <0.05 was considered statistically significant.

3. Results

3.1. MRPA amplification in SbR Leishmania parasites

The molecular karyotype of antimony-resistant and -susceptible *Leishmania* lines, obtained through PFGE is presented in Fig. 1A. Overall, the pattern of the physical chromosomal map obtained for Sb-resistant and -susceptible *Leishmania* parasites is similar except for LgSbR, which, in comparison with its parental WT strain, showed a supplementary band of approximately 1900 kb (Fig. 1A white arrow). The pattern of hybridization of the chromosomes showed that the *MRPA* gene probe recognized a chromosomal band of approximately 800 kb in all lines of *Leishmania* analyzed (Fig. 1B) which fits with the size of chromosome 23 (795 kb). In a preliminary analysis, the intensity of this band was increased in the SbIII-resistant line of *L. amazonensis*. An interesting observation in the antimony-resistant line of *L. braziliensis* (LbSbR) is that the *MRPA* gene probe recognized in this sample a DNA smear and two other bands of approximately 200 kb and 1500 kb (Fig. 1B). This result indicates an extrachromosomal amplification of *MRPA* gene in LbSbR. In agreement with this observation, another band of approximately 700 kb was observed in the resistant sample of *L. infantum* (Fig. 1B).

Southern blot assays were carried out using samples of genomic DNA from different *Leishmania* lines previously digested with the endonuclease *Bam*HI, which has one restriction site within *MRPA* gene (*LbrM23_V2.0280*) in a conserved region. Hybridization of nylon membranes containing *Bam*HI-digested DNA against a *MRPA*-specific probe revealed a major band of 11.0 kb and other faint band of 5.0 kb in all samples analyzed (Fig. 2A). Considering the 11 kb band, after normalization using a *DNA polymerase* gene probe (Fig. 2B), the densitometry revealed an increased intensity of three- and ten-fold in antimony-resistant *L. amazonensis* and *L. braziliensis* lines, respectively, when compared to their susceptible counterparts. This data confirms *MRPA* gene amplification in these antimony-resistant *Leishmania* lines. No difference was observed in the other *Leishmania* lines analyzed.

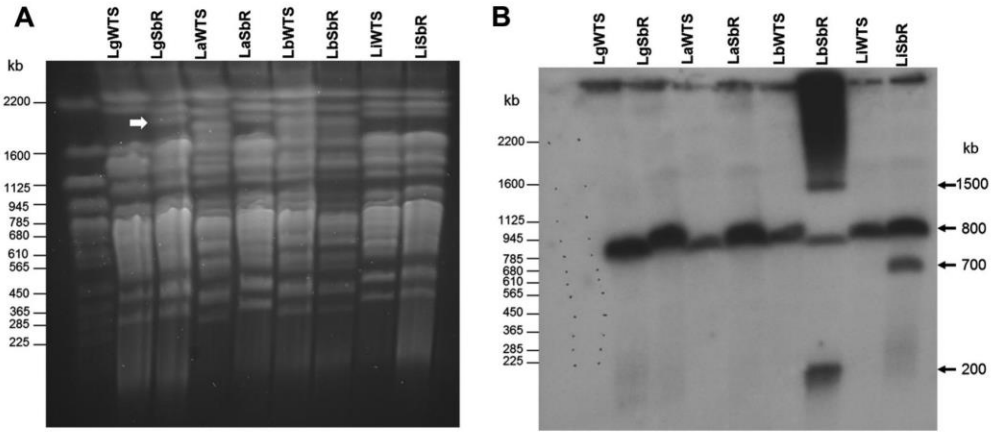

Fig. 1. Chromosomal location of the *MRPA* gene in the antimony-susceptible (WTS) and -resistant (Sb-R) lines of *Leishmania*. (A) Chromosomal bands from the *Leishmania* species were separated by PFGE and stained with ethidium bromide. (B) Profile of the chromosomal bands hybridized with a ^{32}P-labelled *MRPA*-specific probe. Whole chromosomes from *Saccharomyces cerevisiae* were used as molecular weight markers. The white arrow shows an additional band of approximately 1900 kb in the LgSbR line.

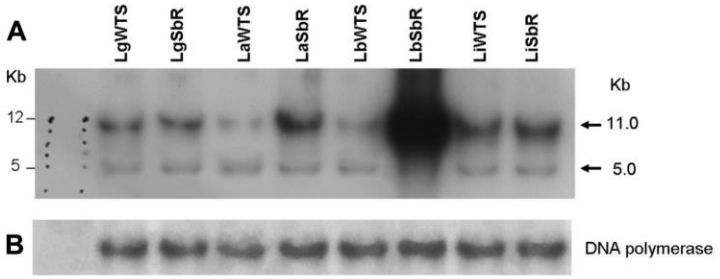

Fig. 2. Southern blot analysis of the *MRPA* gene from antimony-susceptible (WTS) and -resistant (Sb-R) *Leishmania* lines. Total DNA were digested with the *Bam*HI (A) endonuclease, subject to electrophoresis on a 1% agarose gel. Southern blots were hybridized with a ^{32}P-labelled *MRPA*-specific probe. As control, the membrane was exposed to a *Leishmania DNA polymerase* gene probe (B).

3.2. Increased MRPA transcripts in SbR Leishmania

MRPA mRNA levels in the *Leishmania* lines were evaluated by real-time RT-PCR. Measurements of total RNA were normalized by comparison with those obtained for the housekeeping gene *DNA polymerase* (*LbrM.16.1600*). Then, a standard curve was obtained using serial dilutions (10^7–10^2 molecules) of plasmids containing the *DNA polymerase* and *MRPA* genes cloned into pCR2.1 TOPO vector. We obtained a standard curve with good linearity for both genes ($r^2 = 0.998$ to *DNA polymerase* and $r^2 = 0.973$ to *MRPA*). Our data showed a slope of −3.78 and −3.60 for the *DNA polymerase* and *MRPA* genes, respectively, indicating high efficiency of PCR (results not shown). The specificity of PCR was analyzed by plots of the temperature-dependent dissociation of the SYBR GREEN dye from the *MRPA* and *DNA polymerase* PCR products. The results revealed that fluorescence was only emitted at one temperature, suggesting that only one PCR product was generated in each reaction (results not shown).

The amount of cDNA amplified in the samples of *Leishmania* spp. was determined by linear regression analysis using the Ct values obtained from the standard curve generated with known amounts of the plasmids of the *MRPA* gene, normalized with *DNA polymerase* values. The results showed that mRNA level from *MRPA* gene is increased two-fold in the antimony-resistant *L. guyanensis*, *L. amazonensis* and *L. braziliensis* lines compared with their respective susceptible pairs. No difference in the *MRPA* gene expression between antimony-resistant and -susceptible lines of *L. infantum* was observed (Fig. 3).

3.3. Expression level of Pgp

We also determined the levels of Pgp in the antimony-susceptible and -resistant *Leishmania* lines by Western blot analysis using a monoclonal antibody (C219). According to manufacturer's instructions (Abcam), this antibody recognizes an internal, highly conserved amino acid sequence: VQEALD and VQAALD, corresponding to the C-terminal and N-terminal regions, respectively, found in both MDR1 and MDR3 isoforms of P-glycoprotein of mammals. The results revealed that the C219 antibody recognized a polypeptide of 170 kDa in some *Leishmania* samples analyzed (Fig. 4B). According to literature data, this polypeptide corresponds to the expected size of Pgp (Cornwell et al., 1987). Interestingly, this antibody detected Pgp only in antimony-resistant *L. guyanensis* and *L. amazonensis* lines, but not in the susceptible ones. This result indicates that Pgp is more expressed in these antimony-resistant *Leishmania* lines, whereas the Pgp levels in the susceptible lines were not high enough for detection. On the other hand, this antibody recognized Pgp in both antimony-susceptible and -resistant *L. infantum* lines. Densitometry using α-tubulin levels for normalization showed the same level of Pgp expression between both *L. infantum* lines (Fig. 4C). Additionally, we observed that the C219 antibody also detected another polypeptide of about 48 kDa in all antimony-susceptible and -resistant *Leishmania* lines analyzed. However, the intensity of this polypeptide was very faint for both *L. braziliensis* lines (Fig. 4B). The presence of this 48 kDa polypeptide can be due to antibody recognition of a common epitope of another protein of *Leishmania* or an original fragment of Pgp. It is described in the literature that this same anti-Pgp C219 antibody recognizes smaller polypeptides named as "Pgp-like" components (Grogl et al., 1991; Murta et al., 2001; Anacleto et al., 2003).

It is important to emphasize that the anti-Pgp antibody did not detect the protein of 170 kDa in any of the two *L. braziliensis* lines. This result may be due to the absence of overexpression of Pgp in the LbSbR line or changes may have occurred in the amino acid sequence of the epitope region of the protein, preventing its

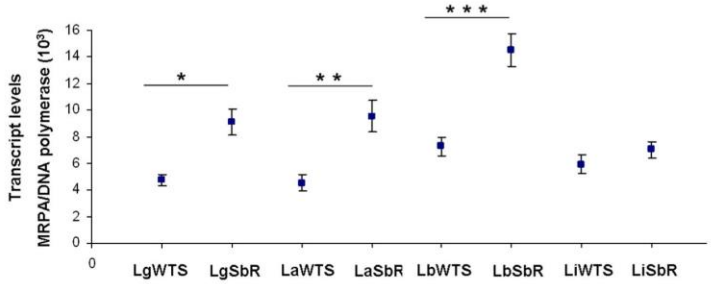

Fig. 3. Levels of transcription of the *MRPA* gene in *Leishmania* spp. susceptible and resistant lines. Levels of *MRPA* mRNA as determined quantitatively (relative to the *DNA polymerase Leishmania* gene) by real-time RT-PCR. Mean values of the transcript levels *MRPA/DNA polymerase* ± standard deviations from three independent experiments are indicated. The mean values for LgWTS and LgSbR, LaWTS and LaSbR and LbWTS versus LbSbR are significantly different (*$p < 0.002$, **$p < 0.003$ and ***$p < 0.001$, respectively), whilst the LiWTS *versus* LiSbR mean values show no difference.

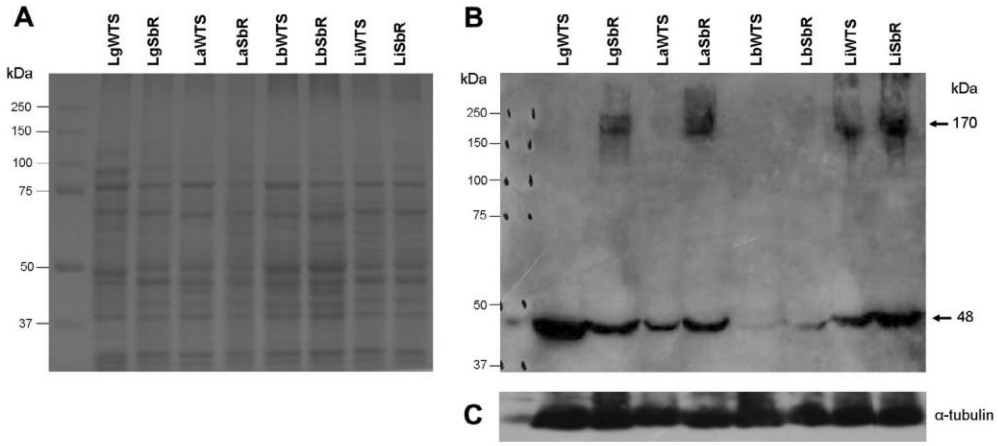

Fig. 4. Pgp expression levels in *Leishmania* species. (A) Electrophoretic profile of proteins from antimony-susceptible (WTS) and -resistant (Sb-R) *Leishmania* lines. Proteins were separated by SDS-PAGE on 12% gel and stained with Coomassie blue. (B) Western blot analysis using the monoclonal antibody C219. (C) The membrane was normalized with the α-tubulin antibody.

recognition by the C219 antibody. We have also questioned whether the protocol used for total protein extraction was appropriate or not to investigate the Pgp expression that is a transmembrane protein. Thus, a protocol described by Grogl et al. (1991) was also used to obtain enriched fraction of membrane proteins separate from soluble protein fraction. After extraction, the membrane proteins fractions of all *Leishmania* lines were submitted to western blot analysis using the C219 antibody. However, the results obtained with this protocol (results not shown) were similar from those previously obtained using total protein (Fig. 4B).

3.4. Intracellular accumulation of antimony

In order to compare the level of antimony uptake between antimony-susceptible and -resistant *Leishmania* lines, the parasites were incubated for 1 h in the presence of 540 μM antimony and, after washing, the intracellular antimony level was quantified by graphite furnace atomic absorption spectrometry. Fig. 5 shows that the uptake of antimony in the resistant lines was significantly reduced in three out of four species analyzed. The data indicate that the antimony levels were approximately seven-, five- and two-fold lower in the antimony-resistant *L. guyanensis* (Fig. 5A), *L. amazonensis* (Fig. 5B) and *L. braziliensis* (Fig. 5C) lines, when compared to their susceptible pairs, respectively. However, the susceptible and resistant lines of *L. infantum* (Fig. 5D) showed no statistically significant difference in the antimony incorporation. The graphics also show marked differences in the level of antimony incorporation between the different species studied.

In addition, these results of antimony intracellular accumulation demonstrate that there is an inverse correlation between the intrinsic antimony susceptibility of the *Leishmania* wild-type lines used in this study and their ability to accumulate antimony. For example, the LgWTS line, which is relatively more Sb sensitive when compared to LiWTS (IC_{50} 0.09 μM versus IC_{50} 0.33 μM, respectively, a 3.7-fold difference), accumulated 14-fold less Sb. The same remark can be made for other lines of *Leishmania*. The LgWTS line accumulated 2.8-fold less Sb than the LaWTS line (IC_{50} 0.28 μM, a 3-fold difference) and the LbWTS line (IC_{50} 0.15 μM) accumulated 5-fold less Sb than the LiWTS line (a 2.2-fold difference in IC_{50}).

3.5. Susceptibility of L. braziliensis lines to BSO and the effect of this inhibitor in the SbIII uptake

We also investigated the effect of BSO, an inhibitor of γ-glutamylcysteine synthetase (GCS) enzyme, in the intracellular accumulation of antimony by LbWTS and LbSbR lines pre-treated with this inhibitor. Interestingly, the BSO susceptibility assay showed that the LbSbR line is more susceptible to BSO than its susceptible counterpart LbWTS. The IC_{50} obtained for LbWTS and LbSbR lines were 15 mM and 2.5 mM, respectively. Then, these lines were pre-treated with BSO during 48 h, incubated with SbIII by 1 h and then submitted to antimony uptake measurement (uptake assay). Our results revealed that LbSbR line accumulated more SbIII compared to its susceptible pair (LbWTS) (Fig. 5E). On the other hand, without BSO, the LbSbR line accumulates less SbIII (Fig. 5C). These results suggest that BSO probably decreases the intracellular concentration of thiols, interfering directly on the SbIII-thiol complex formation and leading to the SbIII accumulation. In addition, we determined the SbIII IC_{50} for the LbWTS and LbSbR lines in the absence and presence of BSO. The LbWTS and LbSbR lines were pre-treated with 15 mM and 2.5 mM BSO, respectively, during 48 h. Subsequently, we incubated these parasites with different SbIII concentrations for 48 h. The IC_{50} obtained for the LbWTS line in the absence of BSO was 0.025 mg/mL and in the presence of BSO was 0.00625 mg/mL (4-fold lower). On the other hand, for the LbSbR line the IC_{50} was 2 mg/mL and after pre-treatment with BSO it was 0.0625 mg/mL (32-fold lower), indicating that the BSO reversed the SbIII resistance phenotype.

3.6. Influx and efflux of antimony

Initially, we performed influx kinetics analysis for the lines of *L. guyanensis* and *L. braziliensis*. Our results showed that both SbR lines exhibited lower rate of influx of SbIII than their respective susceptible counterparts, the difference being much more pronounced in the case of *L. guyanensis* species (Figs. 6A and B). In order to compare the efflux of Sb between susceptible and resistant lines, cells were first loaded with about the same level of antimony. Subsequently, the parasites were washed with HG buffer and aliquots were taken at the times 0, 1 and 2 h to analyze the efflux of antimony by graphite furnace atomic absorption spectrometry.

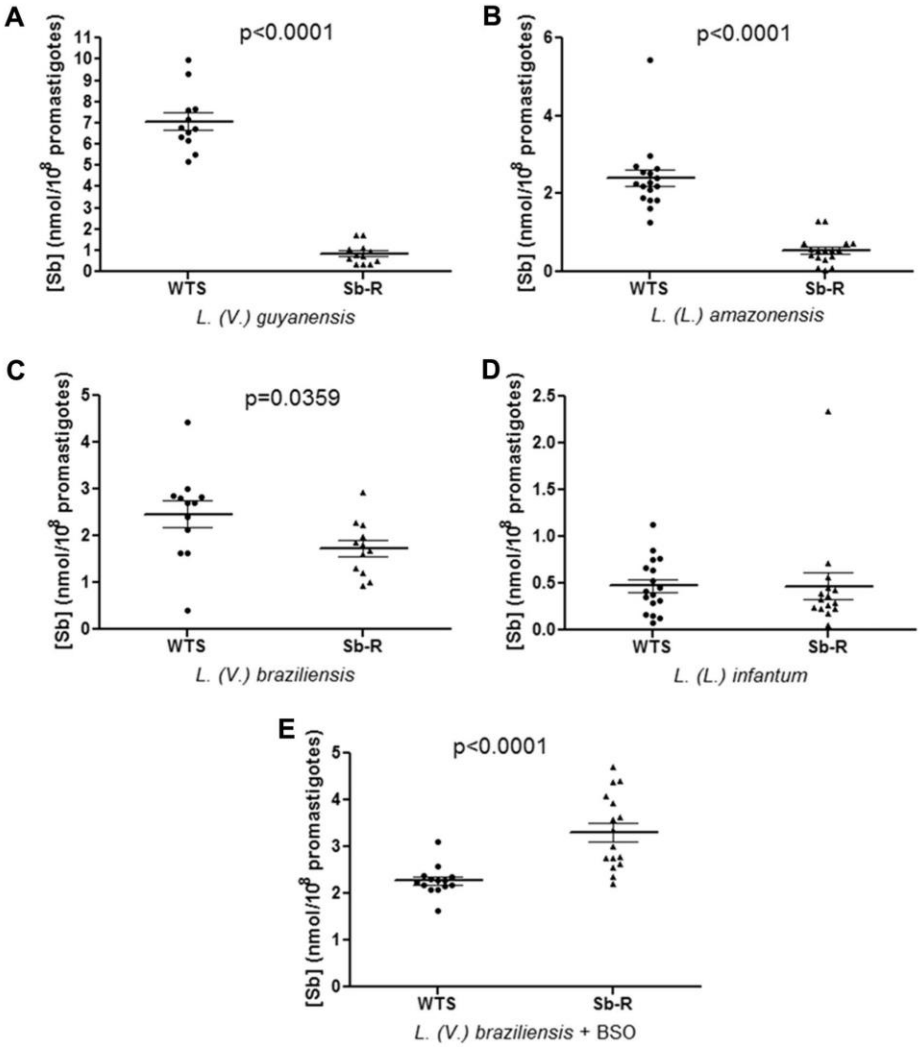

Fig. 5. Antimony uptake in promastigote forms of antimony-susceptible (WTS) and -resistant (Sb-R) lines from four different species of *Leishmania*. Intracellular antimony was quantified by graphite furnace atomic absorption spectrometry after incubation with 540 μM SbIII for 1 h. The values obtained from three independent experiments in quadruplicate, the mean antimony concentration ± SEM and the value *p* are represented in the graphics for the species: *L. (V.) guyanensis* (A), *L. (L.) amazonensis* (B), *L. (V.) braziliensis* (C) e *L. (L.) infantum* (D). The effect of BSO in the SbIII uptake by parasites pre-treated with this inhibitor was also investigated in both *L. (V.) braziliensis* lines (E).

The results demonstrate that the rates of SbIII efflux were higher in both antimony-resistant lines of *L. guyanensis* and *L. braziliensis*, when compared their susceptible pairs (Fig. 6C and D, respectively). This efflux data explains, at least in part, the lower SbIII concentration found in both SbIII-resistant lines, as presented in the Fig. 5A and C, respectively.

3.7. Decreased expression of AQP1 in SbR Leishmania

In order to investigate whether the lower level of intracellular antimony in the SbIII-resistant lines was due to down-regulation of AQP1 protein, we evaluated the expression level of this protein in the four *Leishmania* species studied. Western blot analyses with membrane proteins fractions were performed using a rabbit polyclonal antibody anti-AQP1. Our results showed that this antibody recognized a polypeptide of approximately 35 kDa in all antimony-susceptible and -resistant lines of *Leishmania* analyzed (Fig. 7). After normalization using an α-tubulin antibody, quantification of the band intensity demonstrated that AQP1 expression level is decreased 1.7 and 3-fold in SbIII-resistant lines of *L. amazonensis* and *L. guyanensis*, respectively, when, compared to their respective susceptible pairs (Fig. 7). These data are consistent with the lowest SbIII accumulation presented by these two lines among the species studied (Fig. 5). On the other hand, the expression level of AQP1 was similar between the SbIII-susceptible and -resistant lines of *L. braziliensis* and *L. infantum* showing band intensities ratio (WT/SbR) values of 0.9 and 0.8, respectively (Fig. 7).

4. Discussion

The phenomenon of resistance to antimonials in *Leishmania* is complex, multifactorial and involves several pathways, which have

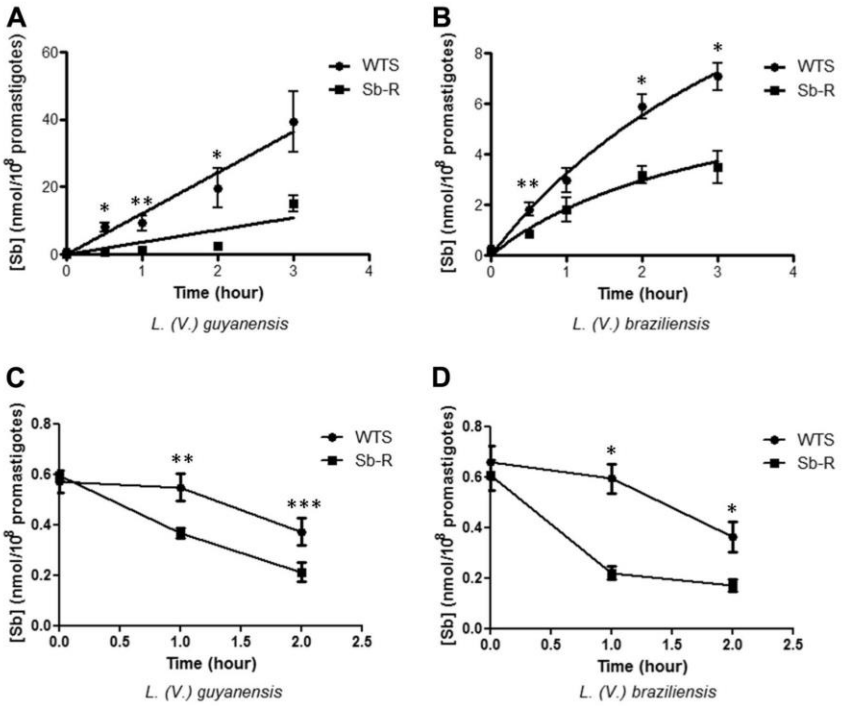

Fig. 6. Time course of SbIII uptake and efflux in promastigote forms from antimony-susceptible (WTS) and -resistant (Sb-R) *Leishmania* lines. The kinetics of uptake and efflux of SbIII were determined by graphite furnace atomic absorption spectrometry in WTS and Sb-R lines of *L. (V.) guyanensis* (A) and *L. (V.) braziliensis* (B) during 0–3 h of SbIII incubation. The efflux rates of *L. (V.) guyanensis* (C) and *L. (V.) braziliensis* (D) were determined during 0–2 h. Data shown are the means of three independent experiments in triplicate of antimony concentration ± SEM. Statistically different values are highlighted as follows: $^{*}p < 0.01$, $^{**}p < 0.02$ and $^{***}p < 0.05$.

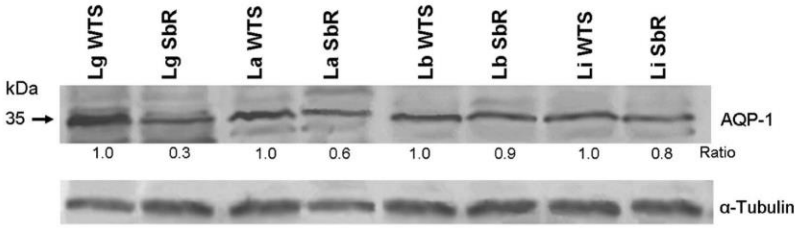

Fig. 7. Expression level of AQP1 protein in *Leishmania* spp. susceptible and resistant lines. Analysis of Western blot with the rabbit polyclonal antibody against the AQP1 protein. The intensity of bands was quantified using ImageJ (http://rsbweb.nih.gov/ij/). Bands were normalized using α-tubulin and the intensity ratio is shown relative to the WTS band (SbR/WTS).

similar features with other microorganisms. These pathways include the entry, metabolism, efflux and/or sequestration, as well as cell death through the drug action (Jeddi et al., 2011). MRPA is one of the most studied ABC transporters in *Leishmania* and its role in the resistance has been well established *in vitro* (Ouellette et al., 1990). However, the majority of these studies were performed using Old World drug-resistant *Leishmania* lines.

Our PFGE results showed that the *MRPA* gene is located in an 800 kb band corresponding to chromosome 23 (795 kb) of *Leishmania*, corroborating literature data (Leprohon et al., 2009; Monte Neto et al., 2011). Interestingly, the *MRPA* gene probe also recognized two other bands of different sizes, 200 and 1500 kb, only in the antimony-resistant *L. braziliensis* line. These supplementary bands indicate that *MRPA* is rearranged or present as an extrachromosomal amplification in LbSbR (Fig. 1B). Additionally, the smear observed exclusively in this resistant line, provide an evidence of circular DNA amplification as indicated by this particular migration in PFGE possibly corresponding to various topoisomers of the circles (White et al., 1988). According to Beverley (1988), large supercoiled circular DNAs appear to exhibit unusual electrophoretic mobility in PFGE, when compared to the large linear chromosomes. Then, an explanation for the presence of approximately 1500 kb chromosomal band in the antimony-resistant *L. braziliensis* line may be related to the integration of this band into large linear chromosomal DNA or the formation of large concatenated networks of circular DNA. Moreover, Grondin et al. (1998) demonstrated the formation of extrachromosomal circular DNA amplification derived from precursors linear amplicons in methotrexate-resistant *L. tarentolae*. This possibility could explain the presence of additional bands concomitant with the smear detected by *MRPA* gene probe in the same LbSbR line. It is also important to note that the *MRPA* gene probe recognized a band of approximately 700 kb only in the antimony-resistant *L. infantum* line. It is described in the literature that the variability in the chromosomal

location can be due to the presence of homologous chromosomes of different sizes, variation in the size of the telomeric regions, small deletions or insertions (Henriksson et al., 1993). However, the formation of a *MRPA*-containing linear extrachromosomal DNA amplification in LiSbR is not discarded. Further studies could confirm the presence of supernumerary chromosomes in these New World *Leishmania* parasites as suggested by *MRPA* hybridization as well as by the presence of a new band in LgSbR compared to LgWTS (Fig. 1A, white arrow). Aneuploidy was a phenomenon previously described in drug-resistant Old World *Leishmania* parasites (Ubeda et al., 2008; Leprohon et al., 2009; Downing et al., 2011) and it supports the evidence of the genome structural plasticity requirement to adapt and survive in the presence of the drug.

Literature data also show the presence of extrachromosomal *MRPA* amplification in other *Leishmania* species. Anacleto et al. (2003) reported the presence of extrachromosomal amplicon of 30 kb containing the PgpA gene in *L. guyanensis* with *in vitro*-induced resistance to Glucantime. Mukherjee et al. (2007) observed that the *MRPA* gene was amplified in clinical isolates of sodium antimony gluconate (SAG)-resistant *L. donovani* lines studied. Recently, Monte Neto et al. (2011) observed that the *MRPA* gene is present in extrachromosomal linear amplicons in two independent mutants of antimony-resistant *L. amazonensis*. Sampaio and Traub-Cseko (2003) reported the presence of amplification of the biopterin transporter in a linear and stable chromosome of 245 kb in *L. braziliensis* (M2903 strain). However, the results obtained by Dias et al. (2007) suggest that in *L. braziliensis* the generation of amplicons is not a common phenomenon when this specie is subjected to drug pressure. Nevertheless, our results in antimony-resistant *L. braziliensis* line indicate that extrachromosomal amplification of the *MRPA* gene may occur, corroborating the data of Sampaio and Traub-Cseko (2003). A possible explanation for the lack of amplification in the samples of *L. braziliensis* analyzed by Dias et al. (2007) may be due to differences in the antimony-resistance level and the protocol used to obtain the resistant parasites when compared to our study.

Our data showed that *MRPA* transcripts are twice as high in the antimony-resistant *L. guyanensis*, *L. amazonensis* and *L. braziliensis* lines, compared to their wild-type pairs. This data correlates with the amplification of this gene in antimony-resistant lines, as shown in PFGE and Southern blot assays. However, the *MRPA* gene amplification of SbIII-resistant *L. braziliensis* line (10-fold higher) does not reflect in the level of mRNA that was only two-fold higher than its susceptible counterpart. This could also be due to post-transcriptional control that decreases the RNA levels of this gene (Teixeira, 1998). It was shown by targeted DNA microarray that the MRPA transporter is overexpressed in axenic amastigotes of antimony-resistant *L. infantum* (El Fadili et al., 2005). Recently, Monte Neto et al. (2011) reported an overexpression of this gene in antimony-resistant *L. amazonensis*, using whole-genome DNA microarrays. Transcriptome data of *L. donovani* from antimony-resistant clinical isolates demonstrated that the *MRPA* gene is not increased in these resistant parasites samples (Decuypere et al., 2005). These differences could be explained by the diversity in resistance indexes as well as in the resistance phenotype stability from different species and genetic background (Decuypere et al., 2012).

A multidrug resistance phenotype associated with the overexpression of Pgp was found in tumoral cell lines and in different pathogenic protozoa (Ullman, 1995). In this study, we investigated whether Pgp, which is a product of the MDR1 gene, was also overexpressed in our antimony-resistant *Leishmania* lines. Our results showed that the anti-Pgp antibody C219 detected a 170 kDa polypeptide corresponding to the expected size for Pgp. Interestingly, this protein is more expressed in the antimony-resistant *L. guyanensis* and *L. amazonensis* lines. However, no difference in Pgp expression between antimony-susceptible and -resistant lines of *L. infantum* was detected, suggesting that Pgp may be not involved in drug resistance in this *Leishmania* species.

In this study, we used the technique of graphite furnace atomic absorption spectrometry to measure the amount of antimony accumulated in the *Leishmania* species. Our data show that the incorporation of antimony was reduced in the antimony-resistant *L. guyanensis*, *L. amazonensis* and *L. braziliensis* lines (Fig. 5). Literature data demonstrated that antimony-resistant *L. infantum* and *L. panamensis* lines incorporated less antimony, when compared to their susceptible pairs (Brochu et al., 2003). Maharjan et al. (2008) also showed that the SAG-resistant clinical isolates of *L. donovani* accumulated about three- to seven-fold less antimony compared to clinical isolates susceptible to this drug. All these literature data corroborate our findings, showing that the resistant parasites accumulate less antimony compared with the respective susceptible pairs.

It has been reported that AQP1 is an important carrier for which cells can accumulate SbIII in *Leishmania* (Borgnia et al., 1999; Gourbal et al., 2004; Marquis et al., 2005). Our results revealed reduced expression of the AQP1 protein in the SbIII-resistant lines of *L. guyanensis* and *L. amazonensis*. Thus, this result may explain, at least in part, the reduced uptake of antimony in these resistant lines of the parasite. In fact, previous studies have shown that the reduced accumulation of SbIII in resistant mutants to this compound could be due to decreased activity of AQP1 and that loss of an allele of this gene can cause an increase in the resistance (Gourbal et al., 2004; Marquis et al., 2005; Mukherjee et al., 2013). It is also possible that another transporter may be involved in the low accumulation of antimony in resistant *Leishmania* lines. On the other hand, no significant difference of antimony incorporation was observed between the antimony-susceptible and -resistant lines of *L. infantum*. This can be explained due to the low resistance index of this antimony-resistant line. These data of antimony accumulation suggest the presence of different antimony-resistance mechanisms among these *Leishmania* species analyzed.

We investigated the SbIII efflux rates in lines of *L. guyanensis* and *L. braziliensis*. The results revealed that the efflux rates are higher in the antimony-resistant lines of these parasites, contributing to the lower accumulation of SbIII in these *Leishmania* species studied. Although, the SbIII-resistant *L. braziliensis* line present low antimony concentration, the level of expression of AQP1 was similar between both SbIII-susceptible and -resistant lines, suggesting that the AQP1 is not involved in the antimony-resistance phenotype. However, the SbIII-resistant *L. braziliensis* line showed an increased rate of antimony efflux, which could explain in part the lower SbIII accumulation in this line.

Interestingly, susceptibility test to BSO, an inhibitor of γ-glutamylcysteine synthetase (GCS), showed that this inhibitor reversed the SbIII-resistance phenotype of *L. braziliensis*. The LbSbR line was more susceptible to BSO than its susceptible counterpart LbWTS. More importantly, with BSO pre-treatment, the resistant parasites LbSbR became 32-fold more susceptible to SbIII than those without treatment. Additionally, we analyzed the effect of the BSO in the uptake of antimony in the lines of *L. braziliensis* pre-treated with this inhibitor. Our results showed that the SbIII-resistant line accumulated more antimony than its wild-type counterpart, suggesting this inhibitor interferes in the polyamine metabolism, changing the intracellular concentration of thiols and antimony. Indeed, *in vitro* studies have shown that BSO, a specific inhibitor of GCS, an enzyme involved in glutathione and trypanothione biosynthesis, can reverse resistance to trivalent antimony in the parasite *L. tarentolae* (Grondin et al., 1997).

In conclusion, our data show that the antimony resistance mechanisms are different in the New World *Leishmania* species analyzed in this study. Functional analysis studies will be performed to investigate the involvement of the *MRPA* gene in our *Leishmania* samples.

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Short communication

Functional analysis of iron superoxide dismutase-A in wild-type and antimony-resistant *Leishmania braziliensis* and *Leishmania infantum* lines

N.G. Tessarollo, J.M. Andrade, D.S. Moreira, S.M.F. Murta *

Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou CPqRR/Fiocruz, Belo Horizonte, MG, Brazil

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ABSTRACT

In this work, we characterized the gene encoding iron superoxide dismutase-A (FeSOD-A) in wild-type (WTS) and antimony-resistant (SbR) *L. (Viannia) braziliensis* and *L. (Leishmania) infantum* lines, which were selected *in vitro*. FeSOD-A transcript and protein expression were similar in all tested lines; however, specific enzyme activity analysis revealed higher superoxide dismutase activity in SbIII-resistant LbSbR and LiSbR lines than in the corresponding WTS lines. These parasites were also more tolerant to oxidative stress generated by the herbicide paraquat. Functional analysis showed that in comparison to non-transfected lines, wild-type LbWTS and LiWTS clones overexpressing the FeSOD-A enzyme are 1.6- and 1.7-fold more resistant to SbIII, respectively. Our results suggest that FeSOD-A is involved in the antimony resistance phenotype in *L. (V.) braziliensis* and *L. (L.) infantum*.

Leishmaniasis is a complex of diseases caused by over 21 different species of unicellular protozoan parasites belonging to the genus *Leishmania*. It is classified by the World Health Organization (WHO) as neglected tropical disease and is a public health problem in many developing countries, particularly in Africa, Latin America, South and Central Asia, the Mediterranean basin, and the Middle East [1]. Leishmaniasis is characterized by a spectrum of clinical manifestations ranging from self-healing cutaneous (CL) and mucocutaneous (MCL) skin lesions to a visceral (VL) form, which is lethal if left untreated. In the New World, *L. (Leishmania) infantum* (syn. *L. (L.) chagasi*) is the causative agent of VL, whereas *L. (Viannia) braziliensis* causes CL and MCL [2].

Pentavalent antimony-containing compounds (SbV) such as sodium stibogluconate (SSG) (Pentostam®) and N-methyl-glucamine (Glucantime®) have been used as first-line therapies against all forms of leishmaniasis for more than 60 years [3]. However, the mechanisms of action of antimony are not entirely clear. It is generally agreed that trivalent antimony (SbIII) is the active form of the drug against amastigote and promastigote forms of *Leishmania* [4]. Some studies have suggested that SbV inhibits macromolecule biosynthesis in amastigotes, possibly via inhibition of glycolysis and fatty acid oxidation [5].

It is well known that *Leishmania* possess an antioxidant defense system for detoxification of reactive oxygen species (ROS) [6] and reactive nitrogen species [7]. The metalloenzyme superoxide dismutase (SOD; EC 1.15.1.1) is a central component in the antioxidant defense in most organisms. It removes excess superoxide radicals (O_2^-) by converting them to oxygen (O_2) and hydrogen peroxide (H_2O_2) [8]. Eukaryotes including mammals have a Cu/Mn/ZnSOD, whereas FeSODs have been found in prokaryotes, protozoans, plants, and algae [8]. Since FeSOD is absent in the human host, this enzyme must be considered as a potential target for chemotherapy against leishmaniasis [9]. Different FeSOD isoforms (FeSOD-A and FeSOD-B) have been characterized in *L. (L.) chagasi*, *L. (L.) tropica*, and *L. (L.) donovani* [10–13]. FeSOD protects the parasite against oxidative stress. According to the TriTryp DataBase, the *L. (V.) braziliensis* (M2904) genome encodes an iron superoxide dismutase *FeSOD-A* gene (LbrM.08.0330) and five putative genes, a *FeSOD-B2* gene (LbrM.32.2010) and four other *SOD* genes (LbrM.30.2740, LbrM.32.2000, LbrM.32.2860 and LbrM.32.2870). The *L. (L.) infantum* (JPCM5) genome encodes a *FeSOD-A* gene (LinJ.08.0300) and four putative genes, a *FeSOD-B1* gene (LinJ.32.1910), a *FeSOD-B2* gene (LinJ.32.1920) and two other *SOD* genes (LinJ.30.2780 and LinJ.32.2770). The FeSOD-A isoform is localized in mitochondria, where occurs numerous chemical reactions involved in cellular respiration producing numerous superoxide radicals as a by-product [12]. The SOD-B1 and SOD-B2 isoforms are present in glycosome of parasite [11].

Several reports have evidenced that the phenomenon of resistance to antimonials in *Leishmania* is complex, multifactorial and may vary considerably between different surveys. It has been demonstrated that resistance is an interplay between uptake, efflux and sequestration of actives molecules [14]. In order to investigate the involvement of FeSOD-A in the antimony-resistance mechanisms of *Leishmania*, we characterized this enzyme in wild-type and antimony-resistant *L. (V.) braziliensis* (MHOM/BR/75/M2904) and *L. (L.) infantum* lines (MHOM/

Abbreviations: SbIII, potassium antimonyl tartrate; WTS, wild-type susceptible; SbR, SbIII-resistant; Lb, *Leishmania (Viannia) braziliensis*; Li, *Leishmania (Leishmania) infantum*; FeSOD, iron superoxide dismutase; qRT-PCR, quantitative real time PCR; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; EC, effective concentration.

* Corresponding author at: Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou CPqRR/Fiocruz, Belo Horizonte 30190-002, MG, Brazil. Tel.: +55 31 33497780; fax: +55 31 32953566.
E-mail address: silvane@cpqrr.fiocruz.br (S.M.F. Murta).

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BR/74/PP75). These lines were selected for *in vitro* resistance to trivalent antimony (SbIII) by step-wise drug pressure; their resistance indices were 20- and 4-fold higher than that of their wild-type counterparts, respectively [15].

The polymorphism and the copy number of the *FeSOD-A* gene in wild-type and SbIII-resistant *Leishmania* lines were analyzed by Southern blotting. The DNA samples were digested with *Hin*fI, which has a single restriction site within *FeSOD-A* (LbrM.08.0330) (Fig. 1S-A Supplementary Material). The *FeSOD-A*-specific probe recognized a major band of 1000 bp in the *L. (V.) braziliensis* lines and a 700 bp band in *L. (L.) infantum* lines. Other low-intensity bands were observed in both lines. Southern blot of *Bam*HI-digested DNA showed a single band of 12 kb in all samples analyzed because there is no restriction site for this enzyme within the reference *FeSOD-A* sequence (Fig. 1S-B Supplementary Material). Paramchuck et al. [10] observed that *LiFeSOD-A* in *L. (L.) infantum* is present in a single copy, while *LiFeSOD-B* may be arranged in multiple copies in the parasite genome. *In silico* analysis of *FeSOD-A* from *L. (V.) braziliensis* (LbrM.08.0330) and *L. (L.) infantum* (LinJ.08.0300) showed that this gene is present in a single copy on chromosome 8 of both species. Southern blot analyses showed species-specific polymorphisms between *L. (V.) braziliensis* and *L. (L.) infantum*. No differences in *FeSOD-A* copy number were observed between wild-type and SbIII-resistant lines of both species, indicating that this gene is not amplified in the genome of SbIII-resistant lines.

Northern blotting and quantitative real time PCR (qRT-PCR) analyses were performed to investigate the levels of *FeSOD-A* mRNA in *Leishmania* lines. Northern blotting with a gene-specific probe revealed the presence of one *FeSOD-A* transcript of approximately 2 kb in all *Leishmania* lines analyzed (Fig. 1A). Paramchuck et al. [10] observed a 1.7 kb transcript in *L. (L.) infantum*. The presence of transcripts larger than the expected size could be related to different levels of mRNA maturation, differences in size of the non-translated 3′ and 5′ regions of the gene or on the size of mRNA poly-A tail [16]. The levels of *FeSOD-A* mRNA in *Leishmania* lines were determined more precisely by qRT-PCR. This procedure was realized as previously described [17], using specific primers (Table 1S Supplementary Material). The amount of *FeSOD-A* cDNA and *18S SSU rRNA* in different *Leishmania* lines was determined by linear regression analysis using the PCR threshold cycle (C_T) values obtained from the standard curve generated with known amounts of the plasmids containing these genes. The amount of *FeSOD-A* cDNA in each line was normalized to that of the housekeeping gene 18 SSU rRNA (LmjF.27.rRNA.01). The results confirmed that the levels of *FeSOD-A* transcription were similar between wild-type and SbIII-resistant *L. (V.) braziliensis* and *L. (L.) infantum* lines (Fig. 1B). Protein expression of FeSOD-A in *Leishmania* lines was evaluated by Western blotting by using a polyclonal antibody raised against a recombinant FeSOD-A protein of *Trypanosoma cruzi* (anti-TcFeSOD-A) [18]. This antibody recognized a 26-kDa polypeptide in all tested *Leishmania* lines (Fig. 1C). Densitometric analysis of this polypeptide compared to the α-tubulin reference (band intensity FeSOD-A/α-tubulin) for each species showed that protein expression was similar between the paired wild-type and SbIII-resistant lines of *L. (V.) braziliensis* and *L. (L.) infantum* (Fig. 1D). Interestingly, this analysis showed also that FeSOD-A protein level was higher in *L. (V.) braziliensis* species when compared with *L. (L.) infantum* (Fig. 1D).

The SOD activity was determined using a simple and rapid method to measure the ability of the enzyme to inhibit the autoxidation of pyrogallol [18,19]. Pyrogallol readily autoxidizes at alkaline pH producing a yellow-brown product detected at 405 nm. SOD activity can be indirectly measured from its ability to inhibit pyrogallol autoxidation. SOD extremely rapid dismutases the superoxide anion radical [20]. Specific enzyme activity analysis of SOD showed significant differences in enzyme activity between wild-type and SbIII-resistant lines of both *Leishmania* species (Table 1). Extracts from SbIII-resistant *L. (V.) braziliensis* (LbSbR) showed 70% SOD activity, whereas those from its wild-type counterpart (LbWTS) showed only 13% SOD activity. Similar results were observed for *L. (L.) infantum*: extracts from SbIII-resistant LiSbR line showed 46% SOD activity and its wild-type counterpart (LiWTS) showed only 1% SOD activity. These results show the importance of this enzyme in the metabolism of SbIII-resistant parasites.

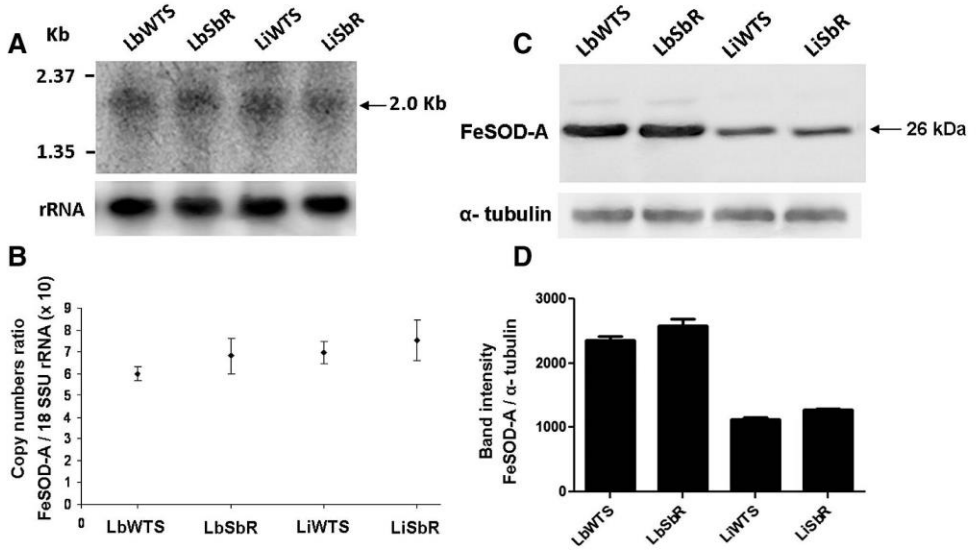

Fig. 1. FeSOD-A gene and protein expression in wild-type and SbIII-resistant *L. (V.) braziliensis* and *L. (L.) infantum*. (A) Northern blot profiles of total RNA extracts obtained using a ^{32}P-labeled FeSOD-A-specific probe [18]; as quantitative control the same membrane was exposed to a *Leishmania* 24S ribosomal RNA probe; and (B) levels of *FeSOD-A* mRNA relative to those of housekeeping gene 18 SSU rRNA determined by real-time RT-PCR. Mean transcript levels for FeSOD-A/18 SSU rRNA ± standard deviations from three independent experiments are shown. WT, wild-type and SbR, SbIII-resistant. (C) Western blot of FeSOD-A. Proteins (20 μg) were separated on a 12% SDS–PAGE gel and blotted onto nitrocellulose membranes. The blots were probed with the rabbit polyclonal antibody against a recombinant FeSOD-A protein of *T. cruzi* (anti-TcFeSOD-A; 1:500) [18] and HRP-conjugated anti-rabbit IgG (1:6000) (GE Healthcare) and developed using ECL Kit (GE Healthcare). The blots were normalized using the anti-α-tubulin monoclonal antibody (1:10,000) (Sigma). (D) Quantification of bands was done by densitometric analysis using the GelAnalyzer2010 software (www.gelanalyzer.com/download.html). The graph shows band intensity (pixels number) FeSOD-A/α-tubulin. Primers are described in Table 1S Supplementary Material.

Table 1
FeSOD enzyme activity of protein extracts from wild-type and antimony-resistant *L. (V.) braziliensis* and *L. (L.) infantum* lines.

Samples	Absorbance (405 nm)[a]	SOD activity (%)[b]
L. (V.) braziliensis		
LbWTS	0.031 ± 0.001	13
LbWTS + pIR1	0.032 ± 0.002	10
LbWTS + pIR1-SOD clone 4	0.003 ± 0.002	83*
LbWTS + pIR1-SOD-clone 5	0.002 ± 0.002	84*
LbSbR	0.008 ± 0.001	70
LbSbR + pIR1	0.005 ± 0.001	60
LbSbR + pIR1-SOD clone 1	0.003 ± 0.001	83
LbSbR + pIR1-SOD clone 2	0.001 ± 0.002	86
L. (L.) infantum		
LiWTS	0.040 ± 0.006	1
LiWTS + pIR1	0.041 ± 0.003	1
LiWTS + pIR1-SOD clone 5	0.031 ± 0.000	13**
LiWTS + pIR1-SOD clone 6	0.024 ± 0.005	29***
LiSbR	0.017 ± 0.001	46
LiSbR + pIR1	0.016 ± 0.000	51
LiSbR + pIR1-SOD clone 1	0.011 ± 0.001	62
LiSbR + pIR1-SOD clone 3	0.009 ± 0.003	67
Controls[c]		
Positive	0.004 ± 0.002	90
Negative	0.041 ± 0.005	0

* $p < 0.0001$, when comparing SOD activity of LbWTS; ** $p < 0.03$ and *** $p < 0.01$, when comparing SOD activity of LiWTS.

[a] Mean value of absorbance at time 5 min subtracted 0 min and the standard deviation of three experiments in duplicate.

[b] Indirect SOD activity (%) was determined from its ability to inhibit pyrogallol auto-oxidation.

[c] Controls – positive: purified bovine SOD enzyme (Sigma) 75 units/200 μL of reaction; negative: without enzyme or extract.

These data are consistent with literature reports in which the *T. cruzi* population having *in vitro*-induced resistance to benznidazole exhibits higher SOD activity than its susceptible counterpart [18]. Interestingly, we observed also differences in SOD activity between the *Leishmania* species analyzed. Elevated SOD activity was detected in *L. (V.) braziliensis* lines when compared to the *L. (L.) infantum* lines (Table 1). This difference can be due to FeSOD-A protein expression level that was higher in *L. (V.) braziliensis* species than in *L. (L.) infantum* (Fig. 1C and D).

Although our Western blot analyses suggested that FeSOD-A expression was similar between SbIII-resistant and wild-type lines, we observed higher SOD activity in both SbIII-resistant *Leishmania* lines. This elevated activity can be due to post-translation modifications of this enzyme, as for example phosphorylation. In addition, in our study all SOD isoforms were evaluated, while protein expression was evaluated only for FeSOD-A, because the antibody used is specific for this isoform.

To investigate the role of FeSOD-A in protecting the parasite against oxidative stress and to verify its possible involvement in the SbIII resistance phenotype, wild-type and SbIII-resistant lines of *L. (V.) braziliensis* and *L. (L.) infantum* were transfected with expression constructs containing *FeSOD-A* (pIR1-BSD-FeSOD) and an empty vector (pIR1-BSD). This procedure was realized as previously described [17]; for details, see the Supplementary Material. The vectors were linearized and electroporated into wild-type and SbIII-resistant *L. (V.) braziliensis* and *L. (L.) infantum* lines. Linearization allows integration of the construct into the ribosomal small subunit locus [21]. Clonal lines resistant to blasticidin (BSD) were selected and analyzed by PCR and western blotting to confirm the presence of the BSD gene and overexpression of FeSOD-A. Western blotting with the polyclonal antibody anti-TcFeSOD-A showed that the transfected parasites overexpressed FeSOD-A (Fig. 2S-A Supplementary Material). Densitometric analysis of the FeSOD-A band, using anti-α tubulin as a reference, showed that the level of FeSOD-A expression was 1.5- to 4-fold higher in transfected clones of wild-type and SbIII-resistant *L. (V.) braziliensis* and *L. (L.) infantum* lines than in the non-transfected or control-transfected derivatives (Fig. 2S-B Supplementary Material).

SOD activity was also measured in the transfected parasites (Table 1). Extracts of the FeSOD-A overexpressing clones 4 and 5 from the wild-type *L. (V.) braziliensis* line showed higher SOD activity (83% and 84%, respectively) than the non-transfected (13%) or control-transfected lines (10%). Extracts of clones 5 and 6 from the wild-type *L. (L.) infantum* line also showed higher SOD activity (13% and 29%, respectively) than the controls (1%). Our results corroborate with those of previous studies showing higher SOD activity in *L. (L.) infantum* lines transfected with SOD-A and SOD-B genes [10]. Interestingly, FeSOD-A overexpressing clones from both SbIII-resistant *L. (V.) braziliensis* and *L. (L.) infantum* lines showed no significant differences in SOD activity (83%–86% for LbSbR and 62%–67% for LiSbR) when compared to non-transfected or control-transfected lines (60%–70% and 46%–51%, respectively). SbIII-resistant parasites showing high SOD activity did not exhibit an altered expression in the presence of FeSOD-A overexpression. These parasites have a very efficient metabolic control system that allows them to avoid any imbalance that could be harmful to the parasite.

Wild-type and SbIII-resistant *L. (V.) braziliensis* and *L. (L.) infantum* lines and FeSOD-A overexpressing clones were incubated with various concentrations of SbIII. The concentration of SbIII required to inhibit the growth by 50% (EC_{50}) was determined using a Z1 Coulter Counter. EC_{50} values were determined from three independent measurements, each performed in triplicate, using the linear interpolation method [22]. Comparative analysis of growth curves of both *Leishmania* species (Fig. 2A and B), showed that those from the *Viannia* sub-genus are more susceptible to SbIII than those from the *Leishmania* sub-genus, as previously demonstrated [15]. As shown in Fig. 2A, increasing concentrations of SbIII produced a decline in the percentage of live parasites from non-transfected (LbWTS) and vector-transfected (LbWTS-pIR1-BSD) lines versus FeSOD-A overexpressing clones, which were more resistant to SbIII. The SbIII EC_{50} of the non-transfected wild-type LbWTS line was 0.044 mg/mL; in contrast, the SbIII EC_{50} of FeSOD-A overexpressing clones 4 and 5 was 0.066 mg/mL and 0.069 mg/mL, respectively. These data show a 1.5- and 1.6-fold increase in the SbIII resistance indices of the FeSOD-A overexpressing clones 4 and 5, respectively (Fig. 2A). Similar results were observed for the wild-type *L. (L.) infantum* line. Gene overexpression increased SbIII resistance. The SbIII EC_{50} values for FeSOD-A overexpressing clones 5 and 6 from LiWTS line were 0.170 and 0.160 mg/mL, respectively, with SbIII resistance indices 1.7- and 1.6-fold higher than that of the non-transfected LiWTS line, respectively (Fig. 2B). No significant difference in SbIII-resistance was observed for FeSOD-A overexpressing clones from both SbIII-resistant *Leishmania* species (data not shown). Similar SbIII EC_{50} was observed in clones from SbIII-resistant FeSOD-A transfected and non-transfected parasites. It is likely that FeSOD-A overexpression did not alter the SbIII-resistance phenotype because these SbIII-resistant parasites already express high levels of SOD activity. We conclude that overexpression of *FeSOD-A* in wild-type lines of *L. (V.) braziliensis* and *L. (L.) infantum* increased resistance to SbIII. Getachew and Gedamu [23] reported the role of LdFeSOD-A in the protection of *L. (L.) donovani* against oxidative stress and in the control of events related to programmed cell death. The authors also showed that overexpression of LdFeSOD-A protects the parasite against cytotoxicity induced by miltefosine and reduces the production of superoxide anion. Recently, Mishra and Singh [24] showed that miltefosine-resistant *L. donovani* lines present increased expression of FeSOD-A. The authors suggest that these parasites have greater mitochondrial protection against oxidative stress, thus inhibiting programmed cell death in the parasites.

The effect of FeSOD-A overexpression on protection against oxidative stress induced by paraquat, a ROS inducer, was evaluated in *L. (V.) braziliensis* and *L. (L.) infantum*. Compared to the controls, the FeSOD-A overexpressing clone 5 from the wild-type *L. (V.) braziliensis* line showed 1.5-fold higher protection against paraquat (Fig. 2C). Statistical analysis showed a significant difference ($p < 0.05$) in the response to 4 and 6 mM paraquat in comparison to non-transfected parasites (LbWTS) (Fig. 2C). However, no difference in the response to 4 mM

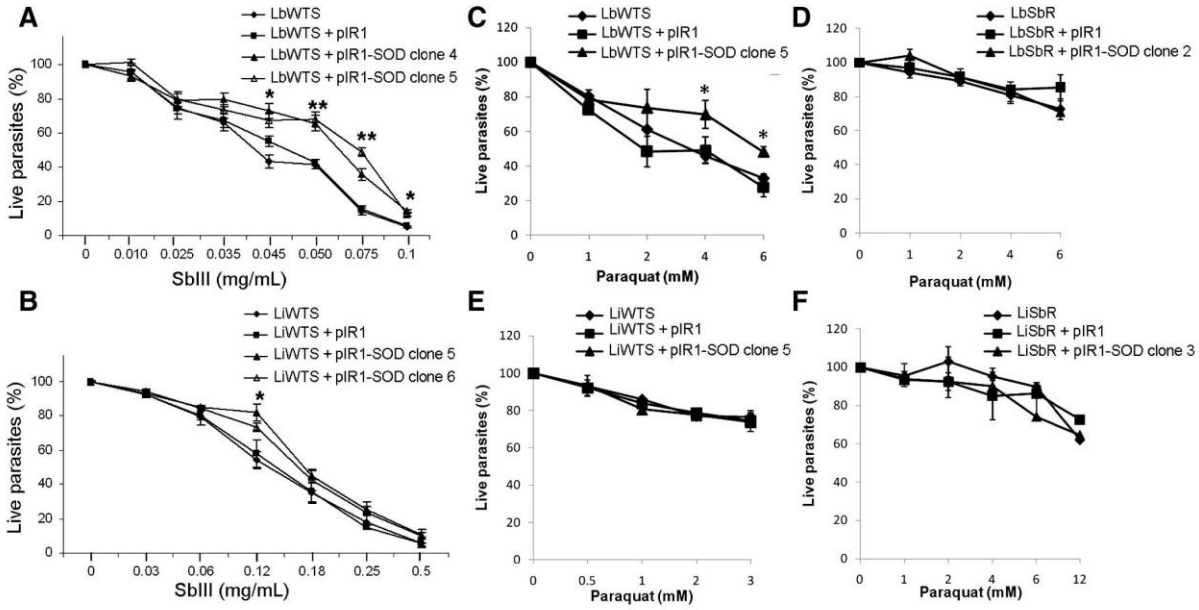

Fig. 2. Susceptibility to SbIII in wild-type *L. (V.) braziliensis* (A) and *L. (L.) infantum* (B) lines transfected with pIR1-BSD or pIR1-BSD-FeSOD-A. Parasites were incubated in the absence or presence of SbIII (0.010–0.25 mg/mL) for 48 h and the percentages of live parasites were determined using a model Z1 Coulter Counter (Beckman Coulter, Fullerton, CA, USA). *In vitro* paraquat tolerance of wild-type (C) and SbIII-resistant (D) *L. (V.) braziliensis* lines and wild-type (E) and SbIII-resistant (F) *L. (L.) infantum* lines transfected with pIR1-BSD or pIR1-BSD-FeSOD-A. Parasites were cultured for 3 or 4 days in the presence of paraquat and the percentages of live parasites were determined using a model Z1 Coulter Counter. Mean values ± standard deviations from three independent (triplicate) experiments are indicated. Data were analyzed by Student's *t* test performed using the software GraphPad Prism 5.0. Statistically different values are denoted as follows: * $p < 0.05$ and ** $p < 0.001$.

paraquat was observed between FeSOD-A overexpressing clone 2 from SbIII-resistant *L. (V.) braziliensis* line and the controls (Fig. 2D). In *L. (L.) infantum*, compared to the controls, both wild-type and SbIII-resistant lines overexpressing FeSOD-A (clones 5 and 3) showed no difference in terms of protection against oxidative stress generated by paraquat (Fig. 2E and F).

Interestingly, we found that the SbIII-resistant lines exhibited greater protection against ROS-induced oxidative stress than their wild-type counterparts did. The percent survival of SbIII-resistant *L. (V.) braziliensis* incubated with 4 mM paraquat was 80%; at the same concentration, only 45% of the wild-type parasites survived ($p < 0.001$) (Fig. 2D and C). Similar results were observed in the wild-type and SbIII-resistant *L. (L.) infantum* lines. At 2 mM paraquat, the percentage of live parasites was 100% for the SbIII-resistant LiSbR line and 77% for its wild-type counterpart, LiWTS ($p < 0.05$) (Fig. 2F and E). This higher tolerance to paraquat exhibited by both SbIII-resistant lines is in agreement with elevated SOD activity in these parasites.

Our studies on the tolerance with oxidative stress induced by paraquat showed that overexpression of FeSOD-A in wild-type *L. (V.) braziliensis* resulted in stronger protection against oxidative stress. Paramchuck et al. [10] observed that *L. (L.) infantum* transfected with isoforms SOD-A and SOD-B were more resistant to paraquat. Ghosh et al. [13] also demonstrated the importance of SOD in protecting *L. (L.) tropica* lines against ROS. Transfection of this gene in the antisense orientation reduced the levels of SOD mRNA and enzymatic activity, thereby increasing sensitivity to menadione (a superoxide anion generator) and hydrogen peroxide [13]. Many studies have shown that there are differences between the antimony-resistant *Leishmania* lines generated in laboratory and clinical isolates, as well as differences intra- and inter-specific of the *Leishmania* parasite. Our results showed elevated activity of superoxide dismutase in SbIII-resistant *L. (V.) braziliensis* and *L. (L.) infantum* lines. Further studies should be performed using antimony-resistant *Leishmania* clinical isolates in order to investigate the role of this enzyme in clinical resistance to antimony.

Intracellular pathogens have developed several mechanisms for the detoxification of ROS. Different enzymes participate in this antioxidant defense: catalase, glutathione peroxidase, superoxide dismutase, and peroxiredoxins. Our proteomic analysis of SbIII-resistant *L. (V.) braziliensis* and *L. (L.) infantum* demonstrated an increased abundance of enzymes involved in antioxidant defense, including pteridine reductase and tryparedoxin peroxidase [25]. In *Leishmania*, FeSOD-A appears to be the first line of defense against ROS. This enzyme is important for parasite survival inside macrophages. Our study suggests that FeSOD-A is involved in the antimony resistance phenotype in *L. (V.) braziliensis* and *L. (L.) infantum*. As this enzyme is absent in humans, it can be considered as a rational target for developing new leishmaniasis therapies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2014.11.001.

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Full length article

Molecular characterization and functional analysis of pteridine reductase in wild-type and antimony-resistant *Leishmania* lines

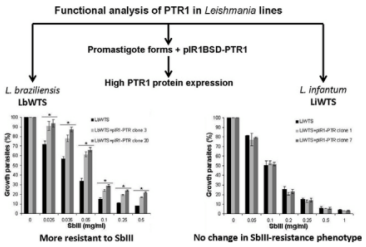
Douglas de Souza Moreira [1], Rafael Fernandes Ferreira [1], Silvane M.F. Murta*

Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou CPqRR/Fiocruz, Av. Augusto de Lima 1715, CEP 30190-002, Belo Horizonte, MG, Brazil

HIGHLIGHTS

- *ptr1* gene is located in a 797 kb chromosomal band in all *Leishmania* lines analyzed.
- PTR1 mRNA and protein levels are increased in the LgSbR, LaSbR and LbSbR lines.
- *Leishmania braziliensis* line over-expressing PTR1 is more resistant to SbIII.
- *Leishmania infantum* line over-expressing PTR1 did not alter the resistance to SbIII.
- PTR1 may be implicated in SbIII-resistance phenotype in *L. braziliensis*.

GRAPHICAL ABSTRACT



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ABSTRACT

Pteridine reductase (PTR1) is an NADPH-dependent reductase that participates in the salvage of pteridines, which are essential to maintain growth of *Leishmania*. In this study, we performed the molecular characterization of *ptr1* gene in wild-type (WTS) and SbIII-resistant (SbR) lines from *Leishmania guyanensis* (Lg), *Leishmania amazonensis* (La), *Leishmania braziliensis* (Lb) and *Leishmania infantum* (Li), evaluating the chromosomal location, mRNA levels of the *ptr1* gene and PTR1 protein expression. PFGE results showed that the *ptr1* gene is located in a 797 kb chromosomal band in all *Leishmania* lines analyzed. Interestingly, an additional chromosomal band of 1070 kb was observed only in LbSbR line. Northern blot results showed that the levels of *ptr1* mRNA are increased in the LgSbR, LaSbR and LbSbR lines. Western blot assays using the polyclonal anti-LmPTR1 antibody demonstrated that PTR1 protein is more expressed in the LgSbR, LaSbR and LbSbR lines compared to their respective WTS counterparts. Nevertheless, no difference in the level of mRNA and protein was observed between the LiWTS and LiSbR lines. Functional analysis of PTR1 enzyme was performed to determine whether the overexpression of *ptr1* gene in the WTS *L. braziliensis* and *L. infantum* lines would change the SbIII-resistance phenotype of transfected parasites. Western blot results showed that the expression level of PTR1 protein was increased in the transfected parasites compared to the non-transfected ones. IC_{50} analysis revealed that the overexpression of *ptr1* gene in the WTS *L. braziliensis* line increased 2-fold the SbIII-resistance phenotype compared to the non-transfected counterpart. Furthermore, the overexpression of *ptr1*

Abbreviations: SbIII, potassium antimonyl tartrate; WTS, Wild-type susceptible; SbR, SbIII-resistant; Lg, *L. (V.) guyanensis*; Lb, *L. (V.) braziliensis*; La, *L. (L.) amazonensis*; Li, *L. (L.) infantum*; PTR1, pteridine reductase 1.

* Corresponding author.
E-mail address: silvane@cpqrr.fiocruz.br (S.M.F. Murta).

[1] Both authors contributed equally.

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gene in the WTS *L. infantum* line did not change the SbIII-resistance phenotype. These results suggest that the PTR1 enzyme may be implicated in the SbIII-resistance phenotype in *L. braziliensis* line.

1. Introduction

Leishmaniasis is a disease complex with wide epidemiological and clinical diversity caused by protozoan parasites belonging to the genus *Leishmania*. They are classified as neglected tropical diseases and responsible for a spectrum of clinical manifestations in humans that include self-healing cutaneous (CL), mucocutaneous (MCL) skin lesions and visceral (VL) form, which is lethal if untreated (Ashutosh et al., 2007). In the New World, *L. (Viannia) guyanensis* and *L. (Leishmania) amazonensis* are the aetiological agents of CL, while *L. (V.) braziliensis* causes CL and MCL, and *L. (L.) infantum* (syn. *L. (L.) chagasi*) is the causative agent of VL (Marzochi and Marzochi, 1994; Murray et al., 2005). The disease is endemic in 98 countries, with an estimated population of 350 million at risk and an incidence of 1.2 million new cases annually (Alvar et al., 2012).

Despite high toxicity and cases of drug resistance, pentavalent antimony-containing compounds (SbV) such as sodium stibogluconate (Pentostam®) and N-methylglucamine (Glucantime®) have been used as first line choice treatment against all leishmaniasis forms for more than six decades (Herwaldt, 1999). The mode of action of antimony is unknown, but it is generally accepted that SbV needs to be reduced within the organism (macrophage or parasite) into the trivalent form (SbIII), which is more toxic and active against the amastigote and promastigote forms of the parasite (Frézard et al., 2001). Earlier studies have been indicated that antimonials probably act by inhibiting glycolysis and fatty acid oxidation (Berman et al., 1987) or by a process of apoptosis involving DNA fragmentation and externalization of phosphatidylserine on the outer surface of membrane of the parasite (Sereno et al., 2001; Sudhandiran and Shaha, 2003). Besides, it has been suggested that SbIII causes disturbances in the thiol redox potential of *Leishmania*, which would lead the cell to death by oxidative stress (Wyllie et al., 2004).

The emergence of resistance to antimonials has been reported, representing a relevant problem in several countries. In India, more than 60% of patients with VL were unresponsive to SbV treatment (Sundar, 2001). The mechanisms of resistance to antimonials have been studied in *Leishmania* species, which include a decrease in the reduction rate from SbV to SbIII and drug uptake, increase in the level of intracellular thiols (cysteine, glutathione and trypanothione) and in the transport (sequestration or efflux) of thiol-metal conjugates (reviewed by Croft et al., 2006).

The pteridine reductase (PTR1) enzyme is an NADPH-dependent reductase that participates in the salvage of pteridines (folate and biopterin), which are essential to maintain growth of *Leishmania* (Nare et al., 2009). This enzyme catalyzes the reduction of folate and biopterin into their biologically active forms tetrahydrofolate and tetrahydrobiopterin, respectively, which act as co-factors (Nare et al., 1997, 2009). Earlier study has been demonstrated that one physiological role of reduced pteridines in *Leishmania* is to deal with oxidative and nitrosative species, and a decreased ability to provide reduced pteridines leads to decreased intracellular survival (Moreira et al., 2009). Indeed, another study performed with *Leishmania major* lines revealed that PTR1 contributes for resistance to oxidative stress within the macrophage, suggesting that the mechanism of action of antimonials might be related to the production of reactive oxygen species (Nare et al., 2009). Thus, as *Leishmania* is auxotrophic for pteridines, a disruption of their salvage process represents a potential therapeutic strategy.

The mechanisms of resistance to antimonials have been extensively studied in Old World *Leishmania* species (Frézard et al., 2014). However, the mechanisms involved in drug resistance in Old and New World pathogenic species are far from being fully elucidated, demonstrating that they are multifactorial and involve different pathways. As recently reported, our proteomic and phosphoproteomic analysis demonstrated that the abundance levels of PTR1 was increased in the SbIII-resistant *Leishmania braziliensis* line compared to its susceptible counterpart (Matrangolo et al., 2013; Moreira et al., 2015). Thus, in this study to investigate better the involvement of this enzyme in the resistance phenotype to trivalent antimony in *Leishmania*, we performed the molecular characterization of PTR1 in wild-type and SbIII-resistant lines of *Leishmania guyanensis*, *Leishmania amazonensis*, *L. braziliensis* and *Leishmania infantum*. Initially, the chromosomal location of *ptr1* gene, mRNA levels and PTR1 protein expression were investigated in these lines. Subsequently, we generated parasites overexpressing this enzyme, which were analyzed for the levels of PTR1 protein expression and susceptibility to SbIII in *L. braziliensis* and *L. infantum* lines.

2. Material and methods

2.1. Leishmania spp. samples

Promastigote forms of *L. guyanensis* (IUMB/BR/85/M9945), *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2904) and *L. infantum* (MHOM/BR/74/PP75) were used in our analysis. The antimony-resistant lines were previously selected *in vitro* to trivalent antimony (SbIII) by step-wise drug pressure and the resistance index varied from 4 to 20-fold higher than of their wild-type counterparts (Liarte and Murta, 2010). Parasites were grown at 26 °C in M199 medium supplemented (Liarte and Murta, 2010). All assays were performed with parasites in the logarithmic phase of growth.

2.2. Pulsed field gel electrophoresis (PFGE)

PFGE assays were performed as previously described (Moreira et al., 2013). Briefly, the agarose blocks containing intact chromosomal DNA from different *Leishmania* lines (2.0×10^9 cells/ml) were separated by PFGE in 1% agarose gels in 0.5x TBE (45 mM Tris–HCl, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 9 °C. The electrophoresis conditions that allowed the separation of the greatest number of parasite chromosomes were: 90 s for 15 h, 200 s for 24 h, 400 s for 15 h and 600 s for 15 h at 90 V. After electrophoresis, bands were transferred onto nylon membrane that was incubated with a ^{32}P-labeled *ptr1* gene probe for identification of the *ptr1* gene. For this probe, was used a 559 bp *ptr1* fragment (LbrM.23.0300) amplified with the primers: forward 5′-TAGATCTCCACCATGACGTCCGTTGCGACAGT-3′ and reverse 5′-GCTGGTTTGTCATGGCGTC-3′.

2.3. Northern blot assays

Total RNA from different *Leishmania* lines was extracted by

RNAzol reagent as described by the manufacturer's instruction (Invitrogen). About 20 μg of total RNA was loaded onto a formaldehyde-agarose gel, stained with ethidium bromide, photographed and transferred onto nylon membrane. Blots were hybridized with [α-^{32}P] dCTP labeled *ptr1* gene probe as described above. *Leishmania 24S ribosomal RNA* gene probe was used as control. The band intensities were analyzed using the software CP ATLAS 2.0 (http://lazarsoftware.com/download.html).

2.4. Western blot analysis

Total proteins from different *Leishmania* lines were obtained as previously described (Gamarro et al., 1994). Protein extracts (40 μg) were separated by electrophoresis on 12% SDS polyacrylamide gel and electrotransferred onto nitrocellulose membranes (BioRad, Hercules, CA, USA). The membranes were blocked by incubation with 5% instant non-fat dry milk in PBS supplemented with 0.05% Tween 20 (PBS-T) for 1 h. The membranes were probed for 12 h at 4 °C in the blocking solution with rabbit polyclonal *L. major* anti-PTR1 antibody (1:100) (kindly provided by Dr. Stephen Beverley, Washington University, USA). The blots were washed and then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) diluted 1:2,000 in blocking solution. After, the blots were washed, incubated with ECL Plus chemiluminescent substrate (GE Healthcare) and exposed to an X-ray film (Amersham, Buckinghamshire, UK). To confirm equivalent loading, SDS-PAGE containing the samples were stained with Coomassie Blue. Furthermore, the anti-α-tubulin monoclonal antibody (1:10,000) (Sigma, St. Louis, USA) was used as standard to normalize the results. The intensity of the bands was analyzed using the software CP ATLAS 2.0.

2.5. Generation of ptr1 overexpressors lines

An 867 bp fragment corresponding to *ptr1* encoding region (LbrM.23.0300) was amplified with *Pfx* DNA polymerase (Invitrogen) from *L. braziliensis* genomic DNA using the forward primer: 5′-TAGATCTCCACCATGACGTCCGTTGCGACAGT-3′ and the reverse primer: 5′-TTAGATCTTCAGGCCCGGGTAAGGCTGTAGC-3′ in which the underlined sequences correspond to *Bgl*II restriction site. The PCR product encoding *ptr1* was cloned into the pGEM T-Easy vector (Invitrogen) and subsequently submitted to sequencing reaction for confirmation of correct sequence. All constructs were sequenced in an ABI 3130 (Applied Biosystems). The pGEM-PTR1 construct was cut with *Bgl*II and the fragment released was subcloned into the dephosphorylated pIR1BSD expression vector (kindly provided by Dr. Stephen Beverley, Washington University, USA). To confirm the correct direction of cloning, the construct was then digested with *Sma*I releasing fragments that confirmed the sense direction of gene. Thereafter, the constructs pIR1BSD (empty vector) and pIR1BSD-PTR1 were linearized by *Swa*I digestion and electroporated into wild-type *L. braziliensis* and *L. infantum* lines. This allows integration of the vector into the ribosomal small subunit locus. Parasite transfection was performed as previously described (Robinson and Beverley, 2003) using a GenePulser XCell (BioRad). Colonies were obtained following plating on semisolid M199 medium containing blasticidin (BSD) (10 μg/ml). After 1–2 weeks, clonal lines were generated and the presence of construct was confirmed by PCR tests using genomic DNA with primers specific for the BSD marker. In addition, Western blot assays were carried out for investigating the level of expression of PTR1 protein in the transfected parasites.

2.6. Susceptibility assay of L. braziliensis and L. infantum clonal lines to SbIII

Promastigotes of wild-type *L. braziliensis* and *L. infantum* clonal lines non-transfected or transfected with the constructs pIR1BSD (empty vector) or pIR1BSD-PTR1 were submitted to SbIII susceptibility assay. Parasites were seeded at 2 x 10^6 cells/ml into 24-well plates in the absence or presence of various concentrations of SbIII (0.025–1.0 mg/ml) for 48 h. The concentration of SbIII required to decrease growth by 50% (inhibitory concentration IC_{50}) was determined using a model Z1 Coulter Counter (Beckman Coulter, Fullerton, CA, USA). IC_{50} values were determined from three independent measurements, each performed in triplicate.

2.7. Statistical analysis

Data were analyzed by Student's t test performed using the software GraphPad Prism 5.0. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Chromosomal location and amplification of the ptr1 gene in Leishmania lines

In this study, we used PFGE assays to evaluate the profile of chromosomal distribution of the *ptr1* gene in *Leishmania* lines (Fig. 1A). The hybridization pattern of the chromosomes demonstrated that the *ptr1* gene probe recognized a 797 kb chromosomal band in all *Leishmania* samples analyzed (Fig. 1B) which fits with the size of chromosome 23 of *Leishmania* (795 kb) (TriTrypDB). In a

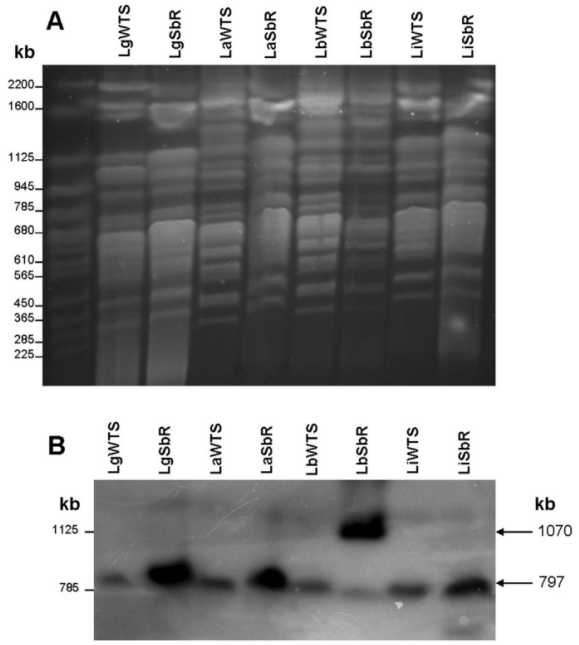

Fig. 1. Chromosomal location of *ptr1* gene in wild-type (WTS) and SbIII-resistant (SbR) *Leishmania* lines. (A) Chromosomal bands from the *Leishmania* lines were separated by PFGE and stained with ethidium bromide. (B) Chromosomal band profiles hybridized with a ^{32}P-labeled *ptr1*-specific probe. Whole chromosomes from *Saccharomyces cerevisiae* were used as molecular weight markers.

preliminary analysis, we observed that the intensity of this band was increased in the SbIII-resistant lines of *L. guyanensis*, *L. amazonensis* and *L. infantum*. Interestingly, we observed that the *ptr1* gene probe also recognized another band of approximately 1070 kb only in the SbIII-resistant *L. braziliensis* line (Fig. 1B), indicating an extrachromosomal amplification of the *ptr1* gene in this LbSbR line.

3.2. Levels of ptr1 mRNA in Leishmania lines

We performed Northern blot analysis to investigate the *ptr1* mRNA levels in *Leishmania* lines. Blots hybridized with *ptr1* gene probe showed the presence of one transcript of approximately 1.6 kb in all *Leishmania* samples analyzed (Fig. 2A). After normalization using a *Leishmania 24S ribosomal RNA* gene probe (Fig. 2B), the results revealed that the levels of *ptr1* mRNA were 3.7-, 2.6- and 4.4-fold higher in the SbIII-resistant *L. guyanensis*, *L. amazonensis* and *L. braziliensis* lines compared to their susceptible counterparts, respectively. No difference was observed between the wild-type and SbIII-resistant *L. infantum* lines (Fig. 2A).

3.3. Levels of PTR1 protein expression

We also determined the levels of PTR1 protein in *Leishmania* lines by Western blot assays using a polyclonal *L. major* anti-PTR1 antibody. The results showed that this antibody recognized a 30.5 kDa polypeptide of expected size in all *Leishmania* samples analyzed (Fig. 3B). Densitometric analysis of this polypeptide compared to α-tubulin levels (Fig. 3C) revealed that the expression level of PTR1 protein is 3- and 4-fold higher in the SbIII-resistant *L. guyanensis* and *L. amazonensis* lines in comparison with their susceptible counterparts, respectively (Fig. 3B). Recently, we observed that PTR1 protein was increased 7-fold in the SbIII-resistant *L. braziliensis* line, whilst the expression level of this polypeptide was similar between the WTS and SbR lines of *L. infantum* (Matrangolo et al., 2013).

3.4. Overexpression of PTR1 enzyme in Leishmania lines and susceptibility assay to SbIII

The constructs containing the *ptr1* gene (pIR1BSD-PTR1) and empty vector (pIR1BSD) were linearized and electroporated into wild-type *L. braziliensis* and *L. infantum* lines. This vector linearized allows integration of the construct into the ribosomal small subunit locus (Robinson and Beverley, 2003). After, the parasite clonal lines resistant to blasticidin (BSD) were selected and subjected to PCR and Western blot assays in order to confirm the presence of the BSD gene and overexpression of the PTR1 enzyme. Colonies of each construct pIR1BSD (empty vector) or pIR1BSD-PTR1 from wild-type

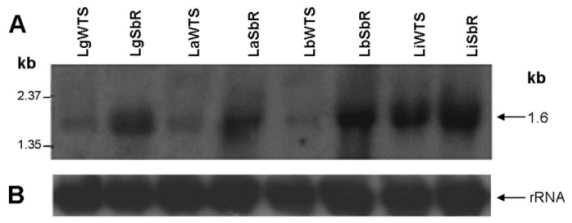

Fig. 2. Northern blot analysis of the *ptr1* gene from wild-type (WTS) and SbIII-resistant (SbR) *Leishmania* lines. (A) Northern blot profiles of total RNA extracts obtained using a ^{32}P-labeled *ptr1*-specific probe. (B) As quantitative control, the membrane was exposed to a *Leishmania 24S ribosomal RNA* gene probe.

L. braziliensis and *L. infantum* lines were evaluated by PCR. The results showed that all colonies resistant to blasticidin analyzed, showed a fragment of 399 bp, corresponding to BSD marker (data not shown). Western blot analysis using a polyclonal antibody, *L. major* anti-PTR1, showed that the transfectant parasites analyzed are overexpressing PTR1 enzyme (Fig. 3D–G). We observed that the level of PTR1 protein expression was 2.3–5.3-fold higher in the transfected clones from wild-type *L. braziliensis* and *L. infantum* lines than in the non-transfected or transfected with empty vector (Fig. 3F–G).

The wild-type lines of *L. braziliensis* and *L. infantum* and *ptr1* overexpressors clones were incubated with different SbIII concentrations. The IC_{50} was determined by counting of parasite number grown in the absence and presence of SbIII. The results demonstrated that the SbIII IC_{50} of the non-transfected wild-type LbWTS line was 0.04 mg/ml. In contrast the SbIII IC_{50} of overexpressors clones 3 and 20 was 0.07 mg/ml and 0.08 mg/ml, which shows an increase of 1.75 and 2.0-fold in the SbII resistance index of these clones, respectively (Fig. 4A). The SbIII IC_{50} of non-transfected wild-type LiWTS line was 0.14 mg/ml, and the SbIII IC_{50} of overexpressors clones 1 and 7 was 0.12 mg/ml and 0.13 mg/ml, respectively (Fig. 4B). This result demonstrates that no difference in SbIII-resistance phenotype was observed for both *ptr1* overexpressors clones from wild-type *L. infantum* line. In all analysis, we also used parasites transfected with empty vector to verify the interference of the vector in our assays. As demonstrated previously, no difference was observed between parasites non-transfected and transfected with empty vector (Andrade and Murta, 2014; Tessarollo et al., 2015).

4. Discussion

There are several hypotheses that may explain the possible mechanism of action of antimonials and one of them is guided in the induction mechanism to oxidative stress generated by SbIII drug (Nare et al., 2009). Thus, the knowledge of the role of the pteridine reductase (PTR1) enzyme in maintaining the levels of tetrahydropteridine seems elucidate one possible mechanism of action of antimonials. This enzyme is responsible for the reduction of pteridines to their active form, tetrahydropteridine. This reduction allows decreasing of the damage caused by reactive oxygen species either by the ability to repair oxidative damage to cellular components or by the maintenance of cellular pathways that affect the oxidative susceptibility (Nare et al., 2009). Moreover, tetrahydropteridine can react rapidly with oxygen, superoxide, peroxynitrite and hydrogen peroxide, demonstrating its important function in protecting of the cells against oxidative damage (Moreira et al., 2009). Literature data emphasize the importance of biopterin as an essential growth factor for *Leishmania* spp. in culture medium. Roy et al. (2001) showed that the genes coding for the PTR1 or for the biopterin transporter (BT1) were overexpressed in *L. major* and *Leishmania donovani* culture-adapted cells in medium with low pterin concentrations. Papadopoulou et al. (2002) demonstrated that biopterin transport in *Leishmania* appears essential, since growth of the *L. donovani* BT1 null mutant was observed only when the medium was supplemented with biopterin. Interestingly, we detected greater survival of *L. braziliensis* and *L. infantum* treated with SbIII in the medium supplemented with biopterin when compared to those parasites grown in the medium without biopterin (data not shown). These results suggest that the biopterin reduced may contribute to decrease the cellular damage caused by action of the SbIII drug and consequently increasing the survival of the parasites. Therefore, due to crucial role of PTR1 enzyme in the antioxidant defense and to be a drug-target candidate for chemotherapy of leishmaniasis, we performed molecular characterization

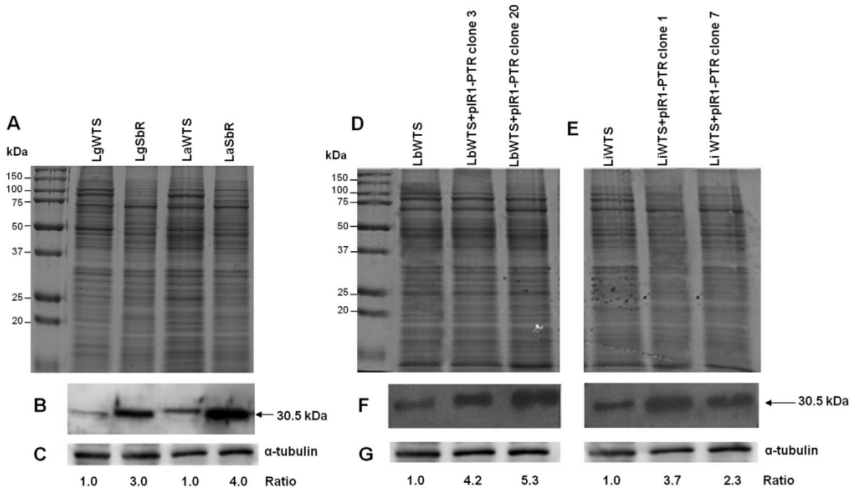

Fig. 3. Levels of PTR1 protein expression in wild-type (WTS) and SbIII-resistant (SbR) *L. guyanensis* and *L. amazonensis* lines, and in clonal lines from wild-type *L. braziliensis* and *L. infantum* non-transfected or transfected with the constructs pIR1BSD-PTR1. Proteins (40 μg) were separated on 12% SDS-PAGE and blotted onto nitrocellulose membranes. (A, D and E) Total protein profile stained with Coomassie blue. (B and F) Western blot analysis using the polyclonal *L. major* anti-PTR1 antibody (1:100) and developed using ECL. (C and G) The membranes were normalized with the α-tubulin monoclonal antibody (1:10,000). The intensity of the bands was analyzed using the software CP ATLAS 2.0.

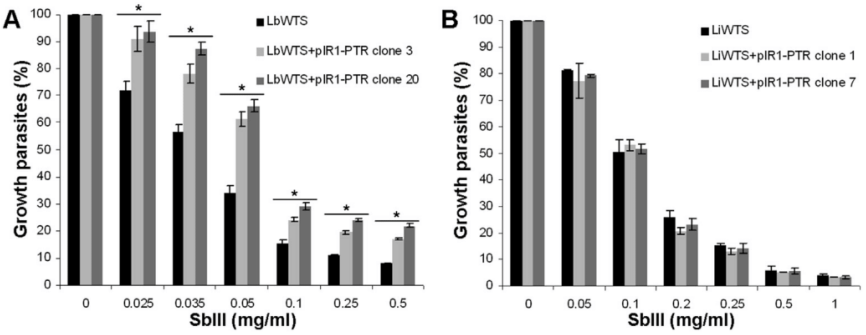

Fig. 4. Susceptibility assay to SbIII of clonal lines from wild-type (WTS) *L. braziliensis* (A) and *L. infantum* (B) lines non-transfected or transfected with the construct pIR1BSD-PTR1. Parasites were incubated in the absence or presence of different concentrations of SbIII (0.025–1.0 mg/ml) for 48 h and the percentages of live parasites determined using a model Z1 Coulter Counter. Mean values ± standard deviations from three independent experiments in triplicate are indicated. Statistically different values are highlighted as follows: $^{*}p < 0.05$ (LbWTS non-transfected line compared to transfected clones).

and functional analysis of this enzyme in wild-type and SbIII-resistant *Leishmania* lines.

Initially, we investigated the chromosomal location and amplification of *ptr1* gene in *Leishmania* lines using PFGE assays. PFGE analysis showed that this gene is located in chromosomal band of approximately 797 kb in all samples analyzed. In LbSbR line, we also observed that the *ptr1* gene probe recognized another chromosomal band of 1070 kb, demonstrating that the *ptr1* gene is amplified in the genome of this SbIII-resistant *L. braziliensis* line. This result indicates that the presence of two chromosomal bands in the LbSbR line may be due to the presence of the *ptr1* gene in homologous chromosomes of different sizes, variation in the size of telomeric regions, small deletions or insertions (Henriksson et al., 1993). Earlier studies have been reported the amplification of the *ptr1* gene in methotrexate-resistant *L. major* and *L. infantum* species (Guimond, 2003; Ubeda et al., 2008), corroborating our data.

The *MRPA* (multidrug resistance protein A) gene is part of the H locus, a region that also contains the *ptr1* gene (Beverley et al., 1984; Ouellette et al., 2002). Recently, our research group demonstrated that the *MRPA* gene is amplified in the SbIII-resistant *L. braziliensis* line (Moreira et al., 2013). This result supports the idea that the *ptr1* gene is co-amplified with *MRPA* gene in our LbSbR line. Interestingly, these both genes were found co-amplified in natural antimony-resistant isolates of *L. donovani* (Mukherjee et al., 2007).

Northern blot data showed that *ptr1* transcritps are higher in the SbIII-resistant *L. braziliensis* line when compared to its susceptible pair. This data is corroborated by earlier microarray results, which demonstrated that the level of *ptr1* mRNA is increased 3-fold in the LbSbR line (Liarte et al. - in preparation). Literature data indicate that the hybridization profile of the *ptr1* mRNA in methotrexate-resistant *L. major* reveals a transcript of 2.4 kb (Guimond, 2003). However, our Northern blot results showed a 1.6 kb transcript in all samples analyzed. One possible explanation for this difference in transcript size may be due to differences in the levels of mRNA maturation with recognition of small mature monocistronic and other polycistronic groups which have not been undergone maturation and processing. Northern blot results also showed that the levels of *ptr1* mRNA are higher in the SbIII-resistant *L. guyanensis*

and *L. amazonensis* lines. Western blot assays demonstrated that the expression level of PTR1 protein is also increased in these both SbR lines. Study previously published by our group showed that the expression level of this protein is higher in the LbSbR line compared to its LbWTS counterpart, while no difference of PTR1 protein was detected between the wild-type and SbIII-resistant lines of *L. infantum* (Matrangolo et al., 2013). Together, our results show that the higher levels of *ptr1*mRNA reflect in the increase in the expression level of PTR1 protein in the SbIII-resistant *L. guyanensis*, *L. amazonensis* and *L. braziliensis* lines. On the other hand, this enzyme is not increased in *L. infantum*, since the data demonstrated similar level of PTR1 protein between the wild-type and SbIII-resistant lines of this parasite. PTR1 protein was found overexpressed in *L. major* methotrexate-resistant mutant, corroborating our results (Drummelsmith et al., 2003).

Functional assays were performed to investigate the role of PTR1 enzyme in the antioxidant defense and to verify its possible involvement in the SbIII-resistance phenotype. For this analysis, we transfected wild-type *L. braziliensis* and *L. infantum* lines with the construction pIR1BSD-PTR1. Western blot results showed that the transfected parasites presented an increase in the expression level of this enzyme compared to their non-transfected pairs. Our data revealed that the wild-type *L. braziliensis* line transfected with the *ptr1* gene increased 2-fold the SbIII-resistance phenotype compared to its respective non-transfected pair. The wild-type *L. infantum* line transfected with the *ptr1* gene did not show difference in the SbIII-resistance phenotype in relation to its non-transfected pair, demonstrating that the overexpression of this gene is not associated with the resistance phenotype to SbIII in this line analyzed. Literature data have been shown that the overexpression of the *ptr1* gene in *Trypanosoma cruzi* lines increases the resistance level to methotrexate and inhibitor drugs of the dihydrofolate reductase enzyme in parasites transfected, when compared to their non-transfected pairs (Robello et al., 1997). Other studies also reported the essential role of PTR1 enzyme in the protection of *Leishmania* against oxidative stress. Moreira et al. (2009) observed an increase in intracellular oxidant molecules in *L. major*, *L. tarentolae* and *L. infantum PTR1*$^{-/-}$ mutants, demonstrating that these parasites were more sensitive to hydrogen peroxide (H_2O_2) and nitric oxide-induced stress. Interestingly, Nare et al. (2009) also showed that *ptr1*$^{-}$ null mutants of *L. major* were 18-fold more sensitive to H_2O_2 than PTR1-overproducing lines, and significant 3–5 fold differences were observed with a broad panel of oxidant-inducing agents.

Recently, we have been shown the involvement of important enzymes such as iron superoxide dismutase and tryparedoxin peroxidase in the antioxidant defense of *Leishmania* parasites (Tessarollo et al., 2015; Andrade and Murta, 2014; Matrangolo et al., 2013). In this study, we can conclude that the PTR1 enzyme may be implicated in the resistance phenotype to SbIII in *L. braziliensis*, since it was demonstrated the protection ability of this protein against oxidative stress in this parasite, supporting the hypothesis that PTR1 can represent a rational target for chemotherapy of leishmaniasis. On the other hand, we observed that this enzyme is not associated to antimony resistance in *L. infantum*. Our previous data demonstrated that SbIII-resistant *L. braziliensis* line present increased expression from *MRPA* gene and reduction in the accumulation of antimony, in contrast no difference was detected in the SbIII-resistant *L. infantum* line compared to its respective SbIII-susceptible line (Moreira et al., 2013). In addition, *L. braziliensis* is more susceptible to SbIII than the *L. infantum* species (Liarte and Murta, 2010) and both present different clinical manifestations and they belong to different subgenus. All these data suggest that different mechanisms of resistance to antimonials may be acting in these *Leishmania* species.

Conflict of interest

The authors have declared no conflict of interest.

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Full length article

Molecular characterization of lipoamide dehydrogenase gene in *Trypanosoma cruzi* populations susceptible and resistant to benznidazole

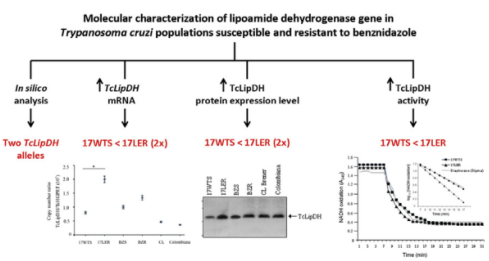
Paula F. dos Santos, Douglas S. Moreira, Elio H. Baba, Caroline M.O. Volpe, Jerônimo C. Ruiz, Alvaro J. Romanha, Silvane M.F. Murta*

Centro de Pesquisas René Rachou, FIOCRUZ, Av. Augusto de Lima 1715, Caixa Postal 1743, CEP 30190-002, Belo Horizonte, MG, Brazil

HIGHLIGHTS

- Lipoamide dehydrogenase (LipDH) gene was characterized in 8 strains and clones of *T. cruzi*.
- *In silico* analysis showed the presence of two *TcLipDH* alleles.
- *TcLipDH* mRNA levels were 2-fold higher in BZ-resistant *T. cruzi* population.
- TcLipDH protein expression level was 2-fold higher in BZ-resistant *T. cruzi* population.
- LipDH activity was higher in BZ-resistant *T. cruzi* population.

GRAPHICAL ABSTRACT



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ABSTRACT

Lipoamide dehydrogenase (LipDH) is a flavin-containing disulfide oxidoreductase from the same group of thioredoxin reductase, glutathione reductase and trypanothione reductase. This enzyme is found in the mitochondria of all aerobic organisms where it takes part in at least three important multienzyme complexes from the citric acid cycle. In this study, we performed a phylogenetic analysis comparing the amino acid sequence of the LipDH from *Trypanosoma cruzi* (TcLipDH) with the LipDH from other organisms. Subsequently, the copy number of the *TcLipDH* gene, the mRNA and protein levels, and the enzymatic activity of the LipDH were determined in populations and strains of *T. cruzi* that were either resistant or susceptible to benznidazole (BZ). *In silico* analysis showed the presence of two *TcLipDH* alleles in the *T. cruzi* genome. It also showed that TcLipDH protein has less than 55% of identity in comparison to the human LipDH, but the active site is conserved in both of them. Southern blot results suggest that the *TcLipDH* is a single copy gene in the genome of the *T. cruzi* samples analyzed. Northern blot assays showed one transcript of 2.4 kb in all *T. cruzi* populations. Northern blot and Real Time RT-PCR data revealed that the *TcLipDH* mRNA levels were 2-fold more expressed in the BZ-resistant *T. cruzi* population (17LER) than in its susceptible pair (17WTS). Western blot results revealed that the TcLipDH protein level is 2-fold higher in 17LER sample in comparison to 17WTS sample. In addition, LipDH activity was higher in the 17LER population than in the 17WTS. Sequencing analysis revealed that the amino acid sequences of the TcLipDH from 17WTS and 17LER populations are identical. Our findings show that one of the

Abbreviations: BZ, benznidazole; LipDH, lipoamide dehydrogenase; Tc, *Trypanosoma cruzi*.

* Corresponding author.
E-mail address: silvane@cpqrr.fiocruz.br (S.M.F. Murta).

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mechanisms associated with *in vitro*-induced BZ resistance to *T. cruzi* correlates with upregulation of LipDH enzyme.

1. Introduction

Chagas disease is caused by the pathogen *Trypanosoma cruzi* and affects about 6–7 million people worldwide (WHO, 2016). Due to intense immigration process this disease have been reported not only in the endemic area, Latin America, but also in North America, Europe and Asia (WHO, 2016) where they are responsible for a global cost of 7 billion dollars annually (Lee et al., 2013).

Chagas disease treatment relies on drugs developed over 45 years ago, nifurtimox (NFX) (5-nitrofuran) and benznidazole (BZ) (2-nitroimidazole) (Perez-Molina et al., 2009). NFX becomes active after its nitro group is reduced to nitro anion radicals that react with oxygen producing toxic oxygen metabolites such as superoxide anions and hydrogen peroxide (Maya et al., 2007). Differently from NFX, BZ trypanocidal effect does not depend on oxygen radicals and previous studies have shown that reduced BZ metabolites bind covalently to macromolecules leading to deleterious effects (Diaz de Toranzo et al., 1988). Metabolomic analysis showed that the binding of BZ reduction products with thiols is responsible by drug toxicity against *T. cruzi* (Trochine et al., 2014). Although BZ and NFX drugs can reach a 76% cure rate in acute cases of Chagas disease, their efficiency is dramatic lower in chronic infections where the percentage of cure is around 8% (Cançado, 2002). This poor efficiency is associated mostly to the existence of naturally resistant *T. cruzi* strains (Filardi and Brener, 1987; Murta et al., 1998; Toledo et al., 2004; Wilkinson et al., 2008) and emphasizes the importance of selecting new drugs and/or targets for Chagas disease treatment.

Drug resistance mechanisms in *T. cruzi* remain poorly understood and several studies have proposed the involvement of different genes which could be associated with resistance to BZ and NFX in this parasite. For instance, one of the mechanisms conferring *in vitro*-induced BZ resistance to *T. cruzi* correlates with deletion of copies of the *TcOYE* gene (Murta et al., 2006). Wilkinson et al. (2008) showed that reduced levels of a type I nitroreductase in an *in vitro*-selected BZ-resistant *T. cruzi* population contributed to the resistance phenotype. In a previous study, we employed Differential Display and Representation of Differential Expression methodologies and demonstrated that lipoamide dehydrogenase (LipDH) was two-fold higher in an *in vitro*-induced BZ-resistant population (17LER) than in its -susceptible counterpart (17WTS) (Murta et al., 2008).

LipDH is a pyridine nucleotide-disulphide oxidoreductase of class I that catalyzes the reaction Dihydrolipoamide + NAD^+ ↔ Lipoamide + NADH + H^+ using FAD as a cofactor (Portela and Stoppani, 1991; Else et al., 1993). This enzyme is a type II nitroreductase found in the mitochondria of all aerobic organisms where it is the third component of the 2-oxo acid dehydrogenase multienzyme complexes from the citric acid cycle.

There are three known 2-oxo acid dehydrogenase multienzyme complexes: (i) the pyruvate dehydrogenase complex, (ii) the 2-oxoglutarate dehydrogenase complex, and (iii) the branched-chain 2-oxo acid dehydrogenase complex (Perham and Packman, 1989; Williams, 1992). The pyruvate dehydrogenase complex is the gateway between glycolysis and the citric acid cycle in aerobic organisms. The 2-oxoglutarate dehydrogenase complex participates in the citric acid cycle and lysine degradation and the branched-chain 2-oxo acid dehydrogenase complex has a role in the metabolism of the branched-chain amino acids leucine, isoleucine and valine. LipDH also takes part in the glycine cleavage system (Walker and Oliver, 1986; Douce et al., 2001) and has an important role in the redox homeostasis, since it is able to catalyze the oxidation of NADH to NAD^+ using different electron acceptors such as oxygen, ferric iron (Petrat et al., 2003), nitric oxide (Igamberdiev et al., 2004), and ubiquinone (Olsson et al., 1999). Reduction of ubiquinone produces ubiquinol, a powerful antioxidant (Nordman et al., 2003). Interestingly, Babady et al. (2007) evidenced that LipDH from mice, pigs and humans also possess a serine protease activity in its homodimer interface.

The studies mentioned above show the role of LipDH in energy and redox metabolism and give evidence that this enzyme can interfere in pathogen infection. Unlike other pathogens, *T. cruzi* has only one isoform of this important enzyme making LipDH a potential drug target. Besides, TcLipDH is part of a group of enzymes with antioxidant activity that is more expressed in an *in vitro*-induced BZ-resistant population, 17LER (Murta et al., 2008). In this study, we performed a phylogenetic analysis comparing the amino acid sequence of the TcLipDH protein with those of LipDH from other organisms. Subsequently, the copy number of the *LipDH* gene, the mRNA and protein levels, as well as the enzymatic activity of the LipDH were determined in populations and strains of *T. cruzi* that were either resistant or susceptible to BZ. We expect that the characterization of this gene in these samples can contribute to elucidate the complex phenomenon of resistance to BZ in *T. cruzi*.

2. Material and methods

2.1. Trypanosoma cruzi populations and strains

In this study, we used a *T. cruzi* population with *in vivo* selected BZ resistance (BZR) and its susceptible pair (BZS) and a pair of BZS and BZR clones (clones 4S and 16R, respectively) (Murta and Romanha, 1998). The BZR *T. cruzi* population was derived from the Y strain, selected *in vivo* after 25 successive passages in mice treated with a single high BZ dose (500 mg/kg of body weight) (Murta and Romanha, 1998). Resistant parasites were maintained in mice treated with this single high dose of BZ at the peak of parasitemia. The mice were bled 6 h after drug administration and the blood was seeded in liver infusion tryptose (LIT) medium at 28 °C to obtain parasite mass. A population with *in vitro*-induced BZ resistance (17LER) and its susceptible pair (17WTS) (Nirdé et al., 1995) were also included in this study. It is important to highlight that the resistance index to BZ of the 17LER parasites (IC_{50} 220 μM) is 23-fold higher than the 17WTS parasites (IC_{50} 9.8 μM). These parasites were kindly provided by Dr. Philippe Nirdé (Génétique Moleculaire des Parasites et des Vecteurs, Montpellier, France). Furthermore, we also used one naturally BZ-resistant strain (Colombiana) and other susceptible strain (CL), previously characterized by Filardi and Brener (1987) regarding their *in vivo* susceptibility to BZ. The epimastigote forms used here were maintained in LIT medium at 28 °C.

2.2. In silico and phylogenetic analyses of the TcLipDH gene

A *TcLipDH* reference sequence (GenBank accession no. X89112) was used in a similarity search against a local copy of the *T. cruzi* database (El-Sayed et al., 2005). Copies of the *T. cruzi* Esmeraldo-like haplotype, Non Esmeraldo-like haplotype and unassigned contigs were downloaded from TriTrypDB (http://tritrypdb.org/common/downloads/release-3.0/Tcruzi/). Search parameters did not include low complexity filters and had an E-value less than $1e^{-6}$.

In order to corroborate our search, positive blast hits (Altschul et al., 1997) were submitted to an *in silico* characterization using a Perl script developed by Dr. Jerônimo C. Ruiz (Ruiz, JC, *personal communication*). This script scanned all TcLipDH protein sequences in search for the motif **G - G - x - C - [LIVA] - x(2) - G - C - [LIVM] – P**. This motif characterizes the active site of the pyridine nucleotide-disulphide oxidoreductases of class I (PDOC00073, E.C 1.8.1.4). In LipDH proteins, the two cysteines participate of the electron transfer from FAD to the lipoamide substrate (Mande et al., 1996). Every residue was confirmed by manual annotation using ARTEMIS (Rutherford et al., 2000).

Phylogenetic analysis was performed as described in Santos et al. (2012). The *TcLipDH* reference sequence (GenBank accession no. X89112) from *T. cruzi* predicted proteome was compared against the proteomes of *Trypanosoma brucei*, three *Leishmania* spp. (*L. braziliensis*, *L. infantum* and *L. major*), one species of algae (*Euglena gracilis*), two species of plants (*Arabidopsis thaliana* and *Lycopersicum esculentum*), and four species of mammals (*Homo sapiens*, *Mus musculus*, *Mesocricetus auratus* and *Cricetulus griseus*). Briefly, the sequences were aligned using MAFFT software (Katoh et al., 2002) and the alignment generated was trimmed using TrimAl (Capella-Gutierrez et al., 2009) to select block of conserved regions. The model JTT was used in the analysis since it was the model which best fitted with the alignment using ProtTest version 2.4 (Abascal et al., 2005). The PHYLIP package version 3.67 was used to perform all other phylogenetic steps (Felsenstein, 2009), and the phylogeny generated in NEXUS format was read in FigTree version 1.2.3 software (http://tree.bio.ed.ac.uk/software/figtree).

2.3. Extraction and preparation of DNA and RNA

T. cruzi genomic DNA and total RNA were extracted and prepared as described previously by Nogueira et al. (2006). For *TcLipDH* gene analysis, 14 μg of genomic DNA from different *T. cruzi* populations and clones were digested with endonucleases *Ava*I and *Eco*RI (Invitrogen, Carlsbad, CA, USA). Southern and Northern blots were hybridized with ^{32}P-labeled *TcLipDH* probes according to Murta et al. (2006).

2.4. Polymerase chain reaction (PCR)

Probes used in Northern and Southern blot assays were prepared from PCR amplification of DNA from *T. cruzi* Y strain. A 500 bp segment of the *TcLipDH* gene (GenBank accession no. X89112) was amplified using the forward primer 5′-ATGTTCCGTCGTTGTGCAGTCAAG-3′ and the reverse primer 5′-TCATCAAACGGCAAGAAGGG-3′. The amplification was carried out in a Perkin Elmer (Waltham, MA, USA) GeneAmp 9600 thermocycler in a final volume of 10 μL containing 0.5 Units of *Taq* DNA polymerase (Invitrogen), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 1X specific *Taq* DNA polymerase buffer together with 10 pmol of each primer. Each reaction was subjected to an initial denaturation at 95 °C for 5 min, followed by 30 cycles of three steps (denaturation at 95 °C for 1 min, annealing 65 °C for 1 min and extension at 72 °C for 1 min). After these cycles, a final extension was performed at 72 °C for 5 min. PCR product was subjected to electrophoresis through a 6% non-denaturing polyacrylamide gel.

2.5. Quantitative real-time RT-PCR

Real time RT-PCR assays were performed as previously described (Nogueira et al., 2006), using the primers forward 5′-CCAACTTTGCACGCTACGG-3′ and reverse 5′- TCATCAAACGGCAAGAAGGG-3′ that amplified a 283 bp segment from the complete nucleotide sequence of *TcLipDH* gene (GenBank accession no. X89112). Standard curves were prepared for each run using known quantities of pCR 2.1-TOPO plasmids (Invitrogen) containing the *TcLipDH* or hypoxanthine-guanine phosphoribosyltransferase (*TcHGPRT*) *T. cruzi* genes. Raw products were quantified using ABI Prism 7000 - Sequence Detection System SDS (PE Applied Biosystems, Foster City, CA, USA). The housekeeping *TcHGPRT* gene, a *T. cruzi* single copy gene, was employed to normalize the amount of each sample assayed (Allen and Ullman, 1994). It is expressed at equivalent levels in all *T. cruzi* samples analyzed presenting a quantitation cycle of 22 ± 0.78.

2.6. Cloning of TcLipDH gene and purification of recombinant protein

The *TcLipDH* complete sequence (GenBank accession no. X89112) was amplified with the forward and reverse primers 5′-CGCGGATCCCCATGTTCCGTCGTTGTGCAGTCAAG-3′ and 5′-CGGGAATTCTTAAAAGTTAATAGTCTTCGCGAC-3′, respectively. The underlined segments added to primers forward and reverse are restriction sites for the enzymes *Bam*HI and *Eco*RI, respectively. These were used to insert the segment into the expression vector pGEX-5X-3 (GE Healthcare). The recombinant vector was transformed into *E. coli* BL-21(DE3)pLysS and the recombinant protein was expressed using 2 mM IPTG (Promega) at 37 °C, under stirring, for up to 6 h. After an SDS-PAGE of total protein from transformed bacteria, the polyacrylamide gel was treated with 0.1 M KCl solution at 4 °C to precipitate the proteins. Subsequently, the band corresponding to the recombinant TcLipDH protein was excised and submitted to electroelution using 3 mL of buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% SDS at pH 8.3) at 100 V for 3 h.

2.7. Production of polyclonal antibody and Western blot assays

The electroeluted fraction containing TcLipDH was used for production of polyclonal antibody. Protocols for immunization, total protein extraction as well as Western blot were performed as described previously (Nogueira et al., 2006). Briefly, total protein from different *T. cruzi* populations and clones were extracted and quantified using Bradford's Method (Bradford, 1976). Following quantification, 20 μg of proteins from each sample were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked, washed and incubated with the polyclonal anti-TcLipDH antibody (1:200). The blots were washed and probed with horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) (1:6000). Subsequently, the membranes were washed, incubated with ECL Plus chemiluminescent substrate (GE Healthcare) and exposed to an X-ray film (Amersham, Buckinghamshire, UK). To normalize the results, the membranes were incubated with the polyclonal *T. cruzi* anti-TcHSP70 antibody. The expression level of TcHSP70 protein has been observed to be equal in both BZ-susceptible and -resistant *T. cruzi* populations (Murta et al., 2008).

2.8. TcLipDH enzymatic assay

Dihydrolipoamide dehydrogenase assay was performed according to Portela and Stoppani (1991) with modifications. For each reaction, 100 μL of a mixture containing 1 mM EDTA, 0.2 mM NADH (Sigma), 1 mM Lipoamide (Sigma) and 50 mM K-phosphate buffer pH 7.5 was submitted to a reading at 340 nm in a Beckman DU 640 Spectrophotometer until a stable baseline was obtained. Subsequently, 35 μg of total protein from *T. cruzi* were added to the cuvette and the absorbance at 340 nm was registered. K-Phosphate buffer and Diaphorase from *Clostridium kluyveri* (Sigma) were used, respectively, as negative and positive controls. Statistical analysis was performed using Mann-Whitney *U* test to compare the results of NADH oxidation between susceptible and resistant *T. cruzi* pairs.

2.9. DNA sequencing

The TcLipDH 1433 bp ORF from *T. cruzi* BZ-susceptible and -resistant populations (17WTS and 17LER) was cloned into the pGEM®-T Easy vector (Invitrogen) and amplified in *E. coli* TOP 10 F′ competent cells. Minipreparations of plasmid DNA were done using the QIAprep Spin Miniprep kit (Qiagen). Aliquots of 500 ng DNA were sequenced using the DYEnamic® ET Dye Terminator Kit (GE Healthcare) in a MegaBACE 1000® DNA Analysis System (GE Healthcare). Sequence analysis was performed according described by Santos et al. (2012).

2.10. Densitometry analyses

All Southern, Northern and Western blot results were photographed and analyzed using ImageMaster VDS software (GE Life Sciences, Little Chalfont, UK). Differences were considered significant when the intensity band ratios were equal to or higher than two-fold.

3. Results

3.1. In silico analyses of TcLipDH gene

Similarity search of the *TcLipDH* sequence (GenBank accession no. X89112) showed one sequence (TcCLB.507089.270) within *T. cruzi* CL Brener Esmeraldo-like haplotype (TcChr35-S) and another (TcCLB.511025.110) within the *T. cruzi* CL Brener Non Esmeraldo-like haplotype (TcChr35-P). These sequences have an identity of 99% between themselves and presented, respectively, 98% and 99% identity when compared to the reference sequence characterized by Schöneck et al. (1997). Also, both of them presented the active site of the pyridine nucleotide-disulphide oxidoreductases of class I (Pyridine Redox I domain, PROSITE documentation: PDOC00073, ExPASy enzyme entry: E.C 1.8.1.4). This active site is identical to the one in human LipDH (Fig. 1S – Supplementary Material). These analyses corroborate the presence of two *TcLipDH* alleles in the *T. cruzi* genome, as stated previously (Schöneck et al., 1997). In addition, to compare the amino acid sequence of TcLipDH with sequences of LipDHs from different organisms (trypanosomatids, plants and mammals), a maximum likelihood phylogenetic tree was constructed (Fig. 1). As expected, it indicated that LipDH from *T. cruzi* grouped with LipDH from *T. brucei* in a cluster near to *Leishmania* genus (average identity over 75%). Furthermore, the LipDH from *T. cruzi* is closer to the LipDHs from the unicellular algae *Euglena gracilis* (60% identity) and plants such as *Arabidopsis thaliana* and *Lycopersicum esculentum* (58% identity) when compared to the homologue enzyme present in mammals (average identity of 50%).

3.2. Copy number of the TcLipDH gene

Southern blot assays were used to analyze the copy number of the *TcLipDH* gene in BZ-susceptible and -resistant *T. cruzi* samples. Genomic DNA was digested with *Ava*I, which has one restriction site within the *TcLipDH* gene sequence, or with *Eco*RI, which has no restriction site within that sequence (GenBank accession no.

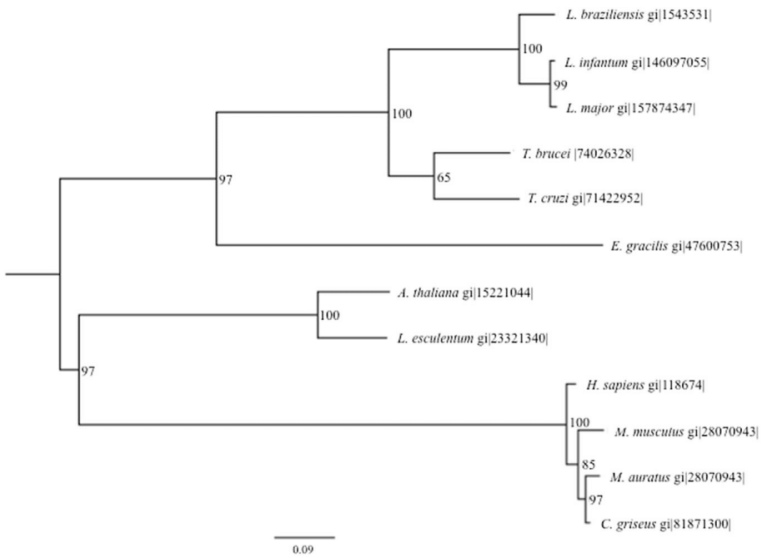

Fig. 1. Maximum likelihood phylogenetic tree based on sequences of lipoamide dehydrogenase of *Trypanosoma cruzi* and other organisms. In the consensus bootstrap tree (1000 replicates) shown, the numbers above each branch represent the bootstrap confidence percentage and the GenBank accession numbers are provided for each species included in the tree.

X89112). The *Ava*I digestion blots showed two bands of 1.9 kb and 0.85 kb (Fig. 2A) while the *Eco*RI digestion blots showed a single band of 5.0 kb (Fig. 2B). These profiles were observed in all of the *T. cruzi* samples analyzed suggesting that the *TcLipDH* is a single copy gene. In addition, densitometry analyses of the blots showed that the intensity of the bands was the same for all *T. cruzi* samples analyzed showing that *TcLipDH* gene is not amplified in the genome of the BZ-resistant *T. cruzi* samples.

3.3. Levels of TcLipDH mRNA in T. cruzi populations

Northern blot profile of total RNA from *T. cruzi* samples hybridized with a specific [^{32}P]dCTP labeled *TcLipDH* gene probe revealed one transcript of 2.4 kb in all the *T. cruzi* samples analyzed (Fig. 3A). Comparative densitometric analyses using a ribosomal RNA (*rRNA*) probe as quantitative control (Fig. 3B) showed that the *TcLipDH* mRNA levels were 2-fold more expressed in the BZ-resistant *T. cruzi* population, 17LER, than in the susceptible pair, 17WTS. No differences in transcript levels were detected between BZS and BZR populations and 4S and 16R clones.

These results were also corroborated by Real Time RT-PCR assays which indicated that the transcript levels of *TcLipDH* are 2-fold higher in 17LER population than in its susceptible counterpart 17WTS (Fig. 3C). No significant differences were observed in the *TcLipDH* mRNA levels between the susceptible and resistant populations of BZS/BZR and CL/Colombiana.

3.4. Levels of TcLipDH protein in T. cruzi populations

In order to compare the levels of TcLipDH in different *T. cruzi* populations, equal amounts of total protein from each population were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The blots revealed that the anti-TcLipDH polyclonal antibody recognized a 50 kDa polypeptide of expected size in all *T. cruzi* samples evaluated (Fig. 4A). For normalization of the results, the same membrane was incubated with polyclonal anti-TcHSP-70 antibody (Fig. 4B). Previous studies performed by our research group have shown that HSP-70 protein is equally expressed in different BZ-susceptible and -resistant samples (Murta et al., 2008) and therefore it was used as quantitative control in densitometry analyses. Densitometry results revealed that TcLipDH protein was 2-fold more expressed in 17LER population in comparison to its respective susceptible pair, 17WTS. No significant difference was observed between the BZS *versus* BZR and CL *versus* Colombiana populations of *T. cruzi*.

3.5. Activity of TcLipDH in T. cruzi populations

Enzymatic activity assay of TcLipDH was performed with the addition of total protein extract from different *T. cruzi* populations to a solution containing lipoamide and NADH. To start the assay, absorbance at 340 nm of the solution containing only lipoamide and NADH was read in average for 7 min until a stable baseline was set. After protein addition, 340 nm absorbance of NADH immediately dropped to minimum indicating substrate exhaustion. The same behavior was observed for the positive control, Diaphorase from *Clostridium kluyveri* (Sigma). Fig. 5 shows the NADH oxidation (A_{340}) of *T. cruzi* extracts from 17WTS and 17 LER populations as function of time. The results showed that LipDH activity of sample 17LER was higher than that of sample 17WTS ($p < 0.05$) during the time period from 8 to 16 min of reaction (Fig. 5). After 16 min of reaction, all samples presented similar profiles indicating that the substrates required for the reaction were no longer available. No difference was observed between the pairs BZS *versus* BZR and CL *versus* Colombiana (data not shown). In addition, we calculated the LipDH specific activity from 17WTS and 17LER extracts (LipDH U/mg of total protein, where units are expressed as Mol product/min). We observed that the LipDH specific activity of 17WTS and 17LER extracts correspond to 4.74 U/mg and 5.31 U/mg, respectively. Based on these results, the extract from 17LER population is 12% more active than its susceptible counterpart 17WTS.

3.6. Sequencing data

DNA sequencing of *TcLipDH* gene from *T. cruzi* susceptible and resistant populations was performed in order to investigate whether point mutations could be causing difference in LipDH activity. Our analysis revealed that the nucleotide and amino acid sequences of TcLipDH from 17WTS and 17LER were identical (Fig. 2S – Supplementary Material).

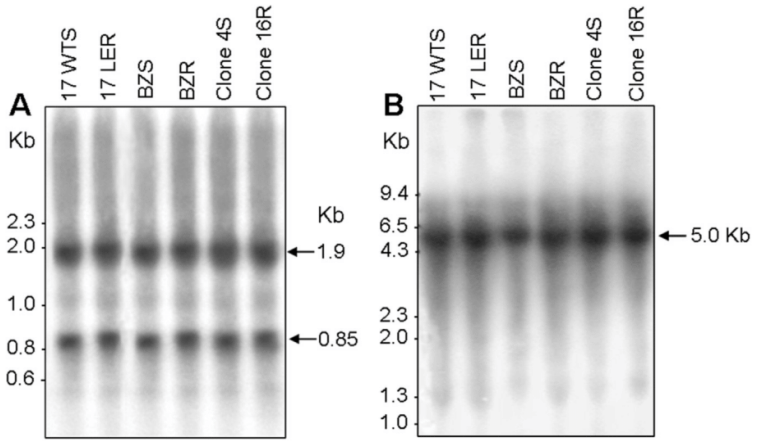

Fig. 2. Southern blot analyses of *TcLipDH* gene from BZ-susceptible and -resistant *T. cruzi* populations. Total DNA (14 μg) was digested with *Ava*I (A) and *Eco*RI (B) endonucleases, subjected to electrophoresis on a 1% agarose gel and hybridized with the 32P-labeled *TcLipDH*-specific probe. The molecular weight markers were derived from λ phage DNA digested with *Hind*III and ΦX 174 DNA digested with *Hae*III.

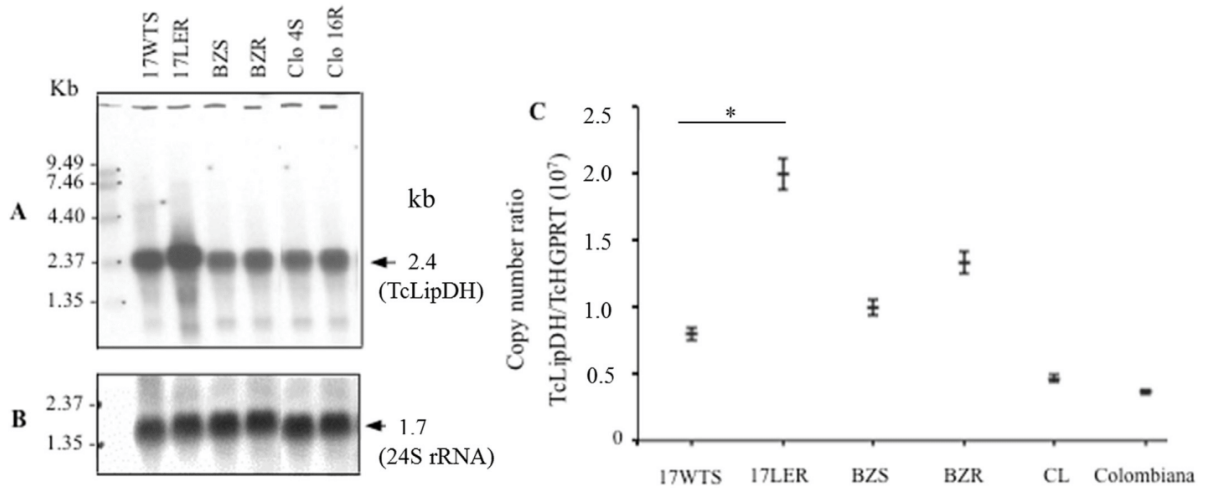

Fig. 3. Transcription levels of the *TcLipDH* gene in BZ-susceptible and -resistant *T. cruzi* populations. (A) Northern blot profiles of total RNA extracts obtained using a ^{32}P-labeled *TcLipDH*-specific probe. (B) As quantitative control, the same membrane was exposed to a *T. cruzi 24S ribosomal RNA* gene probe. (C) Levels of *TcLipDH* mRNA as determined quantitatively (relative to the single-copy housekeeping gene *TcHGPRT*) by quantitative real-time RT-PCR. Mean values of the copy number ratio *TcLipDH*/*TcHGPRT* ± standard deviations from three independent experiments are indicated. The mean values for 17WTS and 17LER are significantly different (*$p < 0.05$), whilst the BZR *versus* BZS and the CL *versus* Colombiana mean values show no difference.

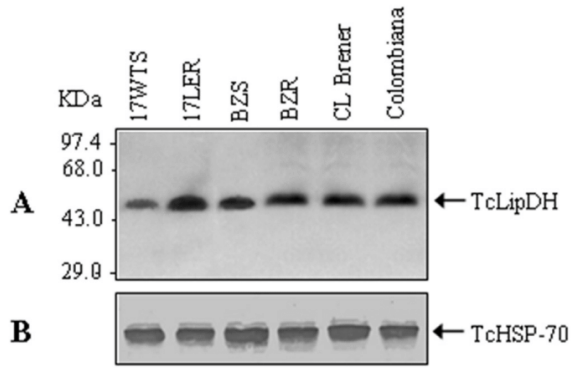

Fig. 4. Levels of TcLipDH protein expression from BZ-susceptible and -resistant *T. cruzi* populations. (A) Proteins (20 μg) were separated on 12% SDS-PAGE, transferred onto nitrocellulose membrane and incubated with the polyclonal *T. cruzi* anti-LipDH antibody. (B) The membrane was incubated with the anti-TcHSP-70 antibody for normalization of the results.

4. Discussion

Lipoamide dehydrogenase (LipDH) is a protein involved in energy and redox metabolism and it is typically located in the mitochondria of aerobic eukaryotes, including the *T. cruzi* (Lohrer and Krauth-Siegel, 1990) and *T. brucei* (Danson et al., 1987; Else et al., 1993). LipDH of *T. brucei* has been shown to play an indispensable role in parasite thymidine biosynthesis, as part of the glycine cleavage complex (Roldán et al., 2011). LipDH is also present in *Mycobacterium tuberculosis* (Venugopal et al., 2011) and *Streptococcus pneumoniae* (Smith et al., 2002), organisms that lack a mitochondria, indicating that this enzyme has functions beyond those related to the known multienzyme complexes. Murta et al. (2008) found that LipDH is part of a group of genes that is more expressed in an *in vitro*-induced BZ-resistant *T. cruzi* population (17LER), using Differential Display and Representation of Differential Expression. In this present study, we have characterized the *LipDH* gene in *T. cruzi* samples with different BZ-resistance phenotypes using *in silico* analysis and molecular assays to investigate gene amplification, mRNA and protein levels.

Our *in silico* analysis revealed that there are two *TcLipDH* sequences in the *T. cruzi* genome presenting the motif that characterizes the active site of the pyridine nucleotide-disulphide oxidoreductases of class I, the Pyridine Redox Domain I. This information is supported by the study of Schöneck et al. (1997) that described the presence of only two alleles of this gene in this parasite. Other studies show evidences of organisms that present more than one isoform of LipDH being this characteristic most frequent in plants, since they express both mitochondrial and plastidial *LipDH* gene (Conner et al., 1996; Lutziger and Oliver, 2000, 2001). In addition to plants, some bacteria (Sokatch, 1981; McCully et al., 1986; Lowe et al., 1983), fungi (Fehrmann and Veeger, 1974; Roy and Dawes, 1987; Kim and Kim, 2010) and the protozoan parasite *Plasmodium falciparum* (McMillan et al., 2005) also present more than one isoform of this enzyme. It is interesting to mention that six other ORFs are annotated as lipoamide dehydrogenases among the unassigned contigs of the *T. cruzi* database. However they are poorly similar to the *TcLipDH* previously described by Schöneck et al. (1997) and they also do not present the characteristic Pyridine Redox Domain I. Other studies would have to be performed to define if those ORFs code for a functional protein.

Considering the two alleles of *LipDH* from *T. cruzi*, a phylogenetic analysis was performed using sequences from other trypanosomatids, mammals and plants. Our analysis showed that *TcLipDH*, as expected, is related to sequences from other trypanosomatids (>75% identity) but is considerably distinct of *LipDH* from *Homo sapiens* (<55% identity). Thus, it is possible that a drug could be designed against *T. cruzi LipDH* without interfering in the metabolism of the mammalian host and that such drug could even be used to treat other diseases caused by trypanosomatids. The challenge of this approach would be to rationally design a drug that did not act via the active site of this enzyme since the active site in both *T. cruzi* and *Homo sapiens* is identical. Another interesting feature of this phylogenetic tree is that *LipDH* from *T. cruzi* is fairly similar to

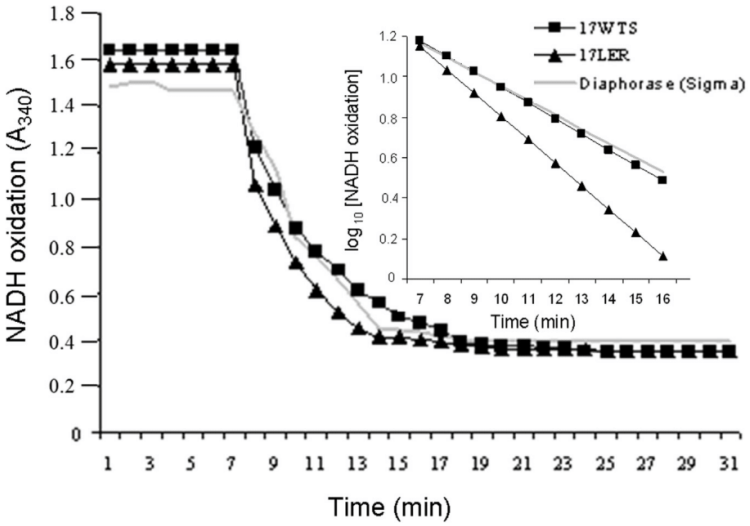

Fig. 5. Comparison of LipDH activity between BZ-resistant (17LER) and -susceptible *T. cruzi* (17WTS) populations showing the NADH oxidation (A340) as a function of time. The results shown are representative of three independent experiments. The means values for 17WTS and 17LER are significantly different ($p < 0.05$) during the time interval from 8 to 16 min. Insert: graphical showing the linear regression of data obtained during the period 7–16 min (log_{10} [NADH oxidation]).

sequences of *LipDH* from the blue algae *Euglena gracilis* and from the plants *Arabidopsis thaliana* and *Lycopersicum esculentum* (>58% identity). Previous studies have reported the existence of several trypanosomatid DNA sequences that are similar to sequences of cyanobacteria and plants due to horizontal transfer among the ancestors of organisms that lived in the same host (Hannaert et al., 2003; Annoura et al., 2005; Opperdoes and Michels, 2007; Týc et al., 2010) and this could be one more example of this DNA exchange.

The *LipDH* mRNA levels were investigated in BZ-resistant and -susceptible samples of *T. cruzi* using Northern blot and Real Time RT-PCR assays. Both methods showed that the *TcLipDH* mRNA levels are two-fold higher in the BZ-resistant population 17LER when compared to 17WTS, its susceptible counterpart. However, according to Southern blot analysis, this gene is not amplified in the genome of these parasites. In fact, all *T. cruzi* strains analyzed revealed a profile compatible with the existence of one allele per haploid genome (Schöneck et al., 1997).

Western blot assay showed that TcLipDH protein was two-fold more expressed in the BZ-resistant population 17LER when compared to its susceptible pair 17WTS. In accordance to our data, a proteomic study conducted by Andrade et al. (2008) using BZ-resistant and -susceptible *T. cruzi* populations and clones also showed that TcLipDH is more abundant in a BZ-resistant clone from a BZ-resistant *T. cruzi* population. In addition, our study showed that the LipDH activity was higher in the 17LER population than in its susceptible counterpart 17WTS. Sequencing analysis revealed that the amino acid sequences of the TcLipDH from 17WTS and 17LER populations are identical. This result suggests that the difference in LipDH activity between 17WTS and 17LER populations is not due to point mutations, but probably due to higher protein expression level.

Literature data support the hypothesis that the overexpression of *TcLipDH* could contribute to the drug resistance phenotype. Diaz et al. (2011) showed that LipDH and tryparedoxin peroxidase were more expressed in amastigotes and trypomastigotes *T. cruzi* forms isolated from acute Chagas disease patients. According to the authors, the overexpression of enzymes involved in the antioxidant defense could favor the survival of the parasite against the oxidative environment of the host. Benznidazole and nifurtimox also produce an oxidative stress, therefore it is also possible that the overexpression of antioxidant enzymes could favor the survival of the parasite in the presence of the drugs (Maya et al., 2007). Previous studies from our group have showed that the *in vitro*-selected BZ-resistant 17LER population overexpressed proteins from antioxidant defense such as iron superoxide dismutase (Nogueira et al., 2006), alcohol dehydrogenase (Campos et al., 2009), cytosolic and mitochondrial tryparedoxin peroxidase (Nogueira et al., 2009) and ascorbate peroxidase (Nogueira et al., 2012).

Villarreal et al. (2004, 2005) showed that there is no correlation between *T. cuzi* groups and drug resistance phenotype, and that different genes are expressed in an *in vivo*-selected and in an *in vitro*-induced BZ-resistant *T. cruzi* strains. Considering that the resistance mechanisms to drugs are complex and multifactorial, *LipDH* and other antioxidant enzymes could confer resistance to the parasite in the oxidative environment promoted by BZ in a resistance model represented by the differentially expressed genes in 17LER population. Therefore, we expect that the characterization of this gene can contribute to elucidate the complex phenomenon of resistance to BZ in *T. cruzi*, and to drive the development of new drugs for chemotherapy against Chagas disease.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exppara.2016.08.006.

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Ornithine decarboxylase or gamma-glutamylcysteine synthetase overexpression protects *Leishmania (Vianna) guyanensis* against antimony

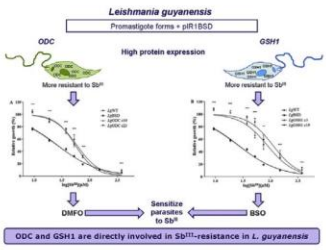
Maisa S. Fonseca [a], Marcelo A. Comini [b], Bethânia V. Resende [a], Ana Maria M. Santi [a], Antônio P. Zoboli [a], Douglas S. Moreira [a], Silvane M.F. Murta [a, *]

[a] Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou CPqRR/Fiocruz, Av. Augusto de Lima 1715 301190-002, Belo Horizonte, MG, Brazil
[b] Laboratorio de Biología Redox de Tripanosomátidos, Institut Pasteur de Montevideo, Mataojo 2020 11400, Montevideo, Uruguay

HIGHLIGHTS

- ODC- or GSH1-overexpressing *L. guyanensis* are more resistant to Sb^{III}.
- DFMO and BSO sensitize LgWT and ODC and GSH1-overexpressors to Sb^{III}.
- DFMO and BSO increase the anti-leishmanial effect of Sb^{III}.
- ODC and GSH1 are implicated in Sb^{III}-resistance in New World *Leishmania*.

GRAPHICAL ABSTRACT



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ABSTRACT

Trypanosomatids present a unique mechanism for detoxification of peroxides that is dependent on trypanothione (bisglutathionylspermidine). Ornithine decarboxylase (ODC) and γ-glutamylcysteine synthetase (GSH1) produce molecules that are direct precursors of trypanothione. In this study, *Leishmania guyanensis odc* and *gsh1* overexpressor cell lines were generated to investigate the contribution of these genes to the trivalent antimony (Sb^{III})-resistance phenotype. The ODC- or GSH1-overexpressors parasites presented an increase of two and four-fold in Sb^{III}-resistance index, respectively, when compared with the wild-type line. Pharmacological inhibition of ODC and GSH1 with the specific inhibitors α-difluoromethylornithine (DFMO) and buthionine sulfoximine (BSO), respectively, increased the antileishmanial effect of Sb^{III} in all cell lines. However, the ODC- and GSH1-overexpressor were still more resistant to Sb^{III} than the parental cell line. Together, our data shows that modulation of ODC and GSH1 levels and activity is sufficient to affect *L. guyanensis* susceptibility to Sb^{III}, and confirms a role of these genes in the Sb^{III}-resistance phenotype.

1. Introduction

Leishmaniasis is a complex of diseases caused by 21–30 different species of protozoan parasites belonging to the genus *Leishmania*. This neglected tropical disease is endemic in 98

Abbreviations: Lg, Leishmania guyanensis; ODC, ornithine decarboxylase; GSH1, gamma-glutamylcysteine synthetase; Sb^{III}, trivalent antimony; DFMO, α-difluoromethylornithine; BSO, buthionine sulfoximine.

* Corresponding author.
E-mail address: silvane@cpqrr.fiocruz.br (S.M.F. Murta).

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countries, especially in Central and South America, Southern Europe, North and East Africa, Middle East, and in the Indian subcontinent (Alvar et al., 2012). Annually, it has been estimated 1.3 million of new cases of the disease and about 30,000 deaths worldwide (WHO, 2016). Leishmaniasis comprises three different main clinical manifestations that range from self-healing cutaneous (CL) and mucocutaneous (MCL) skin lesions to a visceral (VL) form that is lethal if untreated (WHO, 2016). In the New World, *Leishmania (Viannia) guyanensis* causes both CL and MCL (Guerra et al., 2011).

The control of leishmaniasis is based on chemotherapy and there is no human vaccine available to date (Kumar and Engwerda, 2014). Pentavalent antimony-containing compounds such as N-methyl-glucamine (Glucantime®) and sodium stibogluconate (SSG) (Pentostam®) are the main drugs used to treat all forms of the disease for almost eight decades (Mohapatra, 2014). The mechanism of action of this drug is not fully understood. It is known that the pentavalent (Sb^V) form needs to be reduced to the active trivalent form (Sb^{III}), which has leishmanicidal effect against both amastigote and promastigote forms of the parasite (Shaked-Mishan et al., 2001). The antimony has been proposed to inhibit glycolysis, fatty acid oxidation (Berman et al., 1987) and trypanothione reductase, the enzyme responsible for sustaining the parasite' redox homeostasis (Cunningham and Fairlamb, 1995). The cytotoxicity of Sb^{III} has also been associated to alterations in the mitochondrial membrane potential that lead to an increase in the production of reactive oxygen species (ROS) and in the influx of Ca^{++} (Mukherjee et al., 2002; Sudhandiran and Shaha, 2003; Mehta and Shaha, 2006).

In the last 25 years, many cases of treatment failure and resistance to antimonials were reported in several countries including Brazil (Oliveira-Neto et al., 1997), Bolivia (Bermúdez et al., 2006) Colombia (Palacios et al., 2001), India (Sundar, 2001), Iran (Sarkari et al., 2016) and Peru (Arevalo et al., 2007). The most extreme situation takes place in Bihar (India) where 50–65% of the patients are unresponsive to Sb^V-treatment (Sundar, 2001). Parasites use a range of mechanisms to acquire resistance to these drugs such as increase in drug efflux/sequestration or decrease in drug uptake, lower rate of drug activation/reduction and gene amplification (Beverley, 1991; Haldar et al., 2011). Several reports link the level and activity of enzymes from the thiol-redox metabolism of *Leishmania* spp. to antimony resistance (Guimond et al., 2003; Mukherjee et al., 2007; Rai et al., 2013). The central molecule of this metabolism is based the low molecular mass dithiol trypanothione (bisglutathionylspermidine), which provides reducing power for several cellular functions including the protection against reactive oxygen species (Olin-Sandoval et al., 2010; Manta et al., 2013). The two building blocks of trypanothione, glutathione and spermidine are provided by the glutathione- and polyamine-biosynthetic pathway, respectively (Manta et al., 2013). γ-Glutamylcysteine synthetase (GSH1) is the first enzyme of the glutathione pathway that produces γ-glutamylcysteine, a direct precursor of glutathione (Meister and Anderson, 1983). An increase of GSH1 mRNA levels have been reported in some *L. tarentolae* samples with *in vitro*-induced resistance to antimony (Guimond et al., 2003) and some Sb^V-resistant *L. donovani* field isolates (Mukherjee et al., 2007; Singh et al., 2014). Amplification of *gsh1* gene was also observed in Sb^V-resistant *L. donovani* field isolates (Mukherjee et al., 2007; Singh et al., 2014) and in *L. tarentolae* mutants selected for resistance to arsenite (As^{III}) or Sb^{III} (Grondin et al., 1997; Haimeur et al., 1999, 2000).

Ornithine decarboxylase (ODC) converts L-ornithine in putrescine that will be transformed in spermidine by spermidine synthase (Müller et al., 2001). Polyamines contribute to parasite growth and in the As^{III}/antimony-resistance in *Leishmania* parasites (Haimeur et al., 1999; Singh et al., 2007; Birkholtz et al., 2011). Gene amplification and increase in the ODC levels were observed in Sb^V-resistant *L. donovani* field isolates (Mukherjee et al., 2007; Rai et al., 2013) and in *L. tarentolae* mutants selected for resistance to As^{III} (Haimeur et al., 1999).

Despite these evidences, the potential role of ODC and GSH1 enzymes in Sb^{III} resistance for the New World *Leishmania* species has not yet been addressed in detail. In the present study we used a genetic and pharmacological approach to dissect the contribution of ODC and GSH1 enzymes from *L. (V.) guyanensis* (a New World *Leishmania* species) to Sb^{III}-resistance.

2. Material and methods

2.1. Parasites

Promastigotes forms of *Leishmania (Viannia) guyanensis* (IUMB/BR/1985/M9945) were grown at 26 °C in M199 medium (Liarte and Murta, 2010). All assays were performed using parasites in the logarithmic growth phase.

2.2. Cloning, expression and purification of recombinant L. guyanensis GSH1

A 2067 bp fragment corresponding to the complete open reading frame (ORF) of the *gsh1* gene (*LbrM.18.1700*) was amplified from *L. guyanensis* genomic DNA using primers: GSH1pQE-31 forward: 5′-CGCGGATCCGATGGGTCTCTTGACAACTGG-3′ and GSH1pQE-31 reverse: 5′-CGCAAGCTTTTATGCGCTGCTCTCCTGTT-3′. The underlined sequences correspond to *Bam*HI and *Hind*III restriction sites, respectively, to facilitate cloning. PCR amplification was carried as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. The PCR amplicons of *gsh1* were digested with restriction enzymes and then inserted into the corresponding sites of the pQE-31 (Qiagen, Valencia, CA, USA). *Escherichia coli* M15 strain transformed with the pQE31-GSH1 construct were cultured for 12 h in the presence of 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG—Promega, Madison, WI, U.S.A.) at 30 °C, harvested and lysed. The His-tagged recombinant GSH1 was purified using nickel-nitrilotriacetic acid (Ni-NTA) coupled to sepharose (Qiagen) columns. The recombinant protein was used to generate polyclonal antiserum in New Zealand white rabbits according to an immunization protocol previously described (Murta et al., 2006).

2.3. Generation of ODC and GSH1 overexpressor cells from L. guyanensis

Fragments of 1911 and 2067 bp corresponding to *odc* (*LbrM.12.0300*) and *gsh1* (*LbrM.18.1700*) encoding regions, respectively, were amplified with *Pfx* DNA polymerase (Invitrogen) from *L. guyanensis* genomic DNA using the forward primer: 5′-TAGATCT**CCACC**ATGATGAAGATGGTTACCGCC-3′ and the reverse primer: 5′-TTAGATCTCTATAAGGCAATAGAGCTCACC-3′ to amplify *odc* and the forward primer 5′-TGGATCC**CCACC**ATGGGTCTCTTGACAACTGG-3′ and the reverse primer: 5′-ATGGATCCTTATGCGCTGCTCTCCTGTTTCTCAGC-3′ to amplify *gsh1*. Bold letters indicate the Kosak sequence and the underlined sequences correspond to *Bgl*II and *Bam*HI restriction sites of *odc* and *gsh1* genes, respectively. The *odc* and *gsh*1 amplicons were cloned into pGEM T-Easy™ vector (Invitrogen) and subsequently submitted to DNA sequencing. The constructs containing *odc* or *gsh1* were digested with *Bgl*II and *Bam*HI, respectively, and the fragments released were introduced into the dephosphorylated pIR1-BSD expression vector, kindly provided by Dr. Stephen Beverley (Washington

University in St. Louis – USA). To confirm the correct cloning, constructs were digested with *Sal*I and *Sma*I for *odc* and *gsh1*, respectively. The constructs, pIR1-BSD (empty vector), pIR1-BSD-ODC and pIR1-BSD-GSH1 were linearized upon *Swa*I digestion, precipitated and transfected into wild-type *L. guyanensis* using a GenePulser XCell (BioRad) electroporator by electroporation as described in Robinson and Beverley (2003). pIR1 vector allows constructs integration into the 18S ribosomal small subunit (SSU) DNA locus (Robinson and Beverley, 2003). Colonies were obtained following plating on semisolid M199 medium containing 10 μg/mL blasticidin (BSD). After 2–3 weeks, clonal lines were selected and the integration of the ectopic genes was confirmed by PCR tests using genomic DNA with specific primers for the BSD marker according previously described (Tessarollo et al., 2015).

2.4. ODC and GSH1 levels

Protein extracts (40 μg) from *L. guyanensis* were obtained as previously described (Gamarro et al., 1994). Proteins were separated by electrophoresis on 12% SDS polyacrylamide gel and electrotransferred onto nitrocellulose membrane (BioRad). The membrane was blocked by incubation with 5% instant non-fat dried milk in PBS with 0.05% Tween 20 (PBS-T) for 1 h. The blots were then washed twice on PBS-T and incubated for 1 h at room temperature in the presence of the antibodies: rabbit polyclonal *L. donovani* anti-*Ld*ODC (1:200) (kindly provided by Dr. Buddy Ulman – Oregon Health & Science University – USA) and for 2 h with rabbit polyclonal *L. guyanensis* anti-*Lg*GSH1 (1:200) (obtained according to item 2.2). The blots were washed with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000) (GE Healthcare) for 1 h at room temperature. The blots were exposed to Amersham™ ECL Prime Western Blotting Detection Reagent (GE Healthcare) according to the manufacturer's instructions and revealed in ImageQuant LAS 4000 (GE Healthcare). The results were normalized using the anti-α-tubulin monoclonal antibody (Sigma, St. Louis, USA). The intensity of the bands was analyzed using the software CP ATLAS 2.0.

2.5. Susceptibility of *L. guyanensis* cell lines to Sb^{III}

Promastigotes of *L. guyanensis* wild-type, *ghs1*-or *odc*-overexpressor cell lines, were seeded at 2×10^6 cells mL^{-1} into 24-wells plates in absence (control) or presence of several concentrations (9.3–374.2 μM) of potassium antimonyl tartrate/trivalent antimony (Sb^{III}) (Sigma Aldrich) for 48 h. The Sb^{III} effective concentration required to decrease growth by 50% (EC_{50}) was determined using Z1 Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

2.6. Pharmacological inhibition tests of ODC and GSH1

Initially, the EC_{50} of the selective ODC and GSH1 inhibitors DL-α-difluoromethylornithine (DFMO) and *L*-buthionine-sulfoximine (BSO) (both from Sigma Aldrich), respectively, was determined for each *Leishmania* cell line as described above. Then 2×10^6 parasites mL^{-1} from each cell line (wild-type, ODC-overexpressor clone 10 and GSH1-overexpressor clone 3) were pre-incubated for 24 h with the corresponding inhibitors added at their EC_{50} values for the wild-type *L. guyanensis* line: 50 μM for DFMO and 10 mM BSO. Next, these same pre-treated cells were seeded in a 24-well plate and added of Sb^{III} at different concentrations (4.7–74.9 μM), incubated for additional 24 h and finally the percentage of relative growth determined by automated cell counting using a Z1 Coulter Counter.

In addition, WT and GSH1-overexpressor (clone 3) cells were seeded at 2×10^6 parasites mL^{-1} (24-wells plate) in culture medium containing BSO (5 mM for the WT line and 5 or 15 mM for the GSH1-overexpressor). After 5 min, Sb^{III} was added at 9.3 μM (Sb^{III} concentration required to decrease growth by 15 a 20% of both lines) to all wells and incubation extended for 48 h. Cell density was assessed as described above.

2.7. Statistical analysis

The EC_{50} values were determined using the dose-response inhibition equation provided with GraphPad Prism 5.0. Statistical analysis of the data from the susceptibility assays were performed with the ANOVA one-way test using Tukey post-test. Student *t*-test was used for independent samples in assays with the inhibitors. All analyses were performed using GraphPad Prism 5.0 software. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. ODC and GSH1 overexpression in *L. guyanensis*

We transfected the wild-type *L. guyanensis* line with the constructs pIR1-*Lg*ODC or pIR1-*Lg*GSH1 to generate transfectants overexpressing ODC or GSH1. In order to confirm the integration of the vectors, clonal cell lines resistant to blasticidin were subjected to PCR using genomic DNA as template and with specific primers for the BSD marker. Five colonies transfected with the construct pIR1-BSD and ten with pIR1-*Lg*ODC or pIR1-*Lg*GSH1 were evaluated. All 25 analyzed colonies presented a fragment of 399 bp, corresponding to the BSD marker (data not shown). These clones were subjected to western blot analysis to evaluate the levels of ODC and GSH1. The polyclonal antibodies anti-*Ld*ODC and anti-*Lg*GSH1 recognized respectively polypeptides of 69 and 77 kDa in all *Leishmania* cell lines analyzed (Fig. 1A and B). Densitometry analysis of the ODC and GSH1 bands using anti-α-tubulin monoclonal antibody as a reference showed that the levels of ODC and GSH1 were about 2-fold higher in the transfected clones than in cells from the wild-type line or transfected with the empty vector (Fig. 1A and B).

3.2. ODC and GSH1 protect *L. guyanensis* against the inhibitory effect of DFMO and BSO

In order to determine whether the transgenic cell lines from *L. guyanensis* express the active forms of ODC and GSH1, the untransfected and transfected parasites were treated with the corresponding enzyme-specific inhibitors DFMO and BSO, respectively. The EC_{50} of DFMO was 2.2-fold higher for the ODC-overexpressor clones (110 μM) compared to the *Lg*WT (50 μM) whereas the EC_{50} of BSO was at least 3-fold higher for the GSH1-overexpressor clones (>30 mM) than for the *Lg*WT cells (10 mM). The level of resistance towards DFMO and BSO displayed by the overexpressor cell lines that present increased content on ODC and GSH1, confirms that the transgenic parasites express the active form of these enzymes and the selectivity of the inhibitors.

3.3. ODC or GSH1-overexpressing parasites are less susceptible to Sb^{III}

In order to investigate whether overexpression of ODC or GSH1 contributes to the antimony-resistance phenotype, cell lines overexpressing ODC or GSH1, and the parental wild-type line transfected or not with the empty vector (controls) were exposed to different concentrations of Sb^{III} (9.3–374.2 μM) during 48 h. The *Lg*WT and *Lg*BSD cell lines displayed an identical susceptibility towards Sb^{III} (Fig. 2A), presenting EC_{50} of 25.9 and 26.4 μM, respectively. In contrast, the clones overexpressing *Lg*ODC and *Lg*GSH1

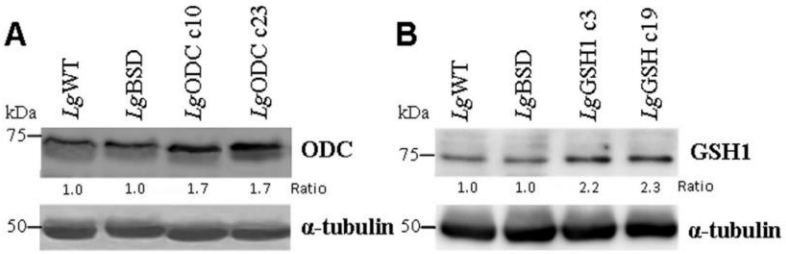

Fig. 1. Expression level of (A) ornithine decarboxylase (ODC) and (B) γ-glutamylcysteine syntethase (GSH1) in *L. (V.) guyanensis*. Western blot from total cell lysates (40 μg protein/lane) separated on a 12% SDS polyacrylamide gel and probed with rabbit polyclonal antibodies anti-*Ld*ODC or anti-*Lg*GSH1 (both diluted 1:200) and HRP-conjugated anti-rabbit IgG (dilution 1:5000). The signals for ODC and GSH1 were normalized using the anti-α-tubulin monoclonal antibody (dilution 1:5000). The *L. guyanensis* cell lines tested are *Lg*WT: wild-type, *Lg*BSD: *Lg*WT transfected with the empty vector, *Lg*ODC c10 or c23: clone 10 or 23 from ODC overexpressor, *Lg*GSH1 c3 or c19: clone 3 and 19 from GSH1 overexpressor.

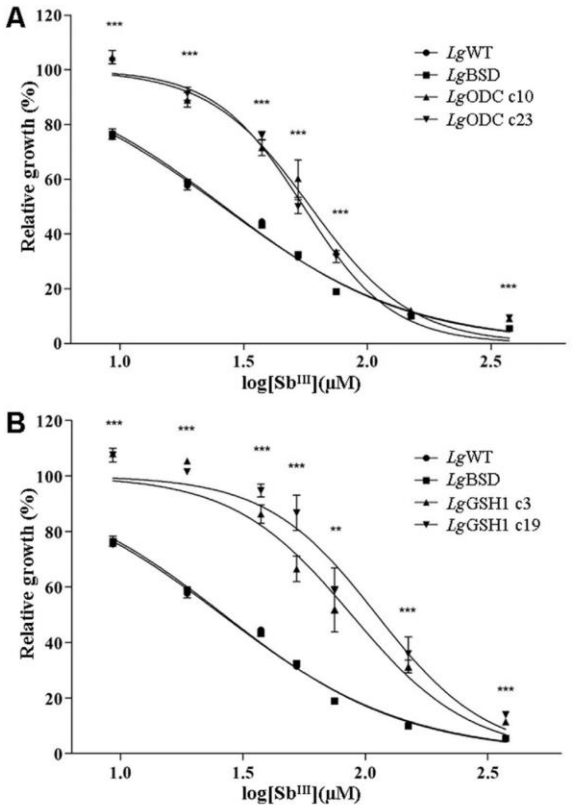

Fig. 2. Susceptibility to Sb^{III} of *L. (V.) guyanensis* cell lines overexpressing (A) ODC and (B) GSH1. Parasites were incubated in M199 medium in the absence or presence of various concentrations of Sb^{III} (9.3–374.2 μM) for 48 h and the percentage of relative growth determined using Z1 Coulter Counter. Mean values and standard deviations from four independent experiments are shown. Statistically different values (ANOVA one-way test, GraphPad Prism 5.0) are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.0001$. *Lg*WT: wild-type, *Lg*BSD: *Lg*WT transfected with the empty vector, *Lg*ODC c10 or c23: clone 10 or 23 from ODC overexpressor, *Lg*GSH1 c3 or c19: clone 3 and 19 from GSH1 overexpressor.

showed a higher resistance against Sb^{III} (Fig. 2A and B). The Sb^{III} resistance indexes determined for the clones 10 and 23 from the *Lg*ODC cell line are about 2-fold higher than that of the *Lg*WT cell line (Table 1), showing EC_{50} of 58.7 and 55.2 μM, respectively. This result is in agreement with the expression level obtained for the ODC transgenic parasites. Interestingly, the *Lg*GSH1 overexpressors clones 3 and 19 presented resistance indexes 3.4–4.3-fold than the *Lg*WT line, presenting EC_{50} of 87.7 and 111.2 μM, respectively (Table 1).

In the pharmacological inhibition tests, the different *L. guyanensis* cell lines were pre-treated during 24 h with 50 μM DFMO or 10 mM BSO (EC_{50} concentration corresponding to the *Lg*WT cell line) and subsequently exposed to different Sb^{III} concentrations for additional 24 h. The results show that treatment with DFMO or BSO reduced drastically parasite growth of *Lg*WT in the presence of Sb^{III}. In contrast, cells overexpressing ODC or GSH1 were significantly more resistant to an identical treatment with the selective inhibitors and Sb^{III} (Fig. 3A and B). For instance, the susceptibility to Sb^{III} increased 648-fold for *Lg*WT and only 1.5-fold for *Lg*ODC (clone 10) when parasites were pre-treated with DFMO (Table 1). Although to a minor extent, BSO treatment increased 20- and 4.1-fold the sensitivity towards Sb^{III} of *Lg*WT and *Lg*GSH1 (clone 3) parasites, respectively (Table 1). These data clearly demonstrate that inhibition of ODC and GSH1 sensitize parasites against antimony and, the opposite, overexpression of these enzymes confers resistance towards this drug.

In addition, the effect of a 48 h-combined treatment with BSO and Sb^{III} on *Lg*WT and *Lg*GSH1 (clone 3) lines was evaluated (Fig. 4). No difference on cell proliferation between *Lg*WT and *Lg*GSH1 lines was observed when 9.3 μM Sb^{III} was added alone (15–22% growth inhibition). We observed a reduction of 20% on cell proliferation of *Lg*WT line and no change in the growth of *Lg*GSH1 line when 5 mM BSO was added individually. On the other hand, an inhibition of approximately 6% was detected on cell proliferation of *Lg*GSH1 line at concentration of 15 mM BSO. Interestingly, for the *Lg*WT parasites, co-incubation with both 9.3 μM Sb^{III} and 5 mM BSO impaired cell growth to a significant extent (88% growth inhibition). In contrast, an identical treatment only halved the proliferation of *Lg*GSH1 cells (45% growth inhibition) and it was necessary to increase 3 times the concentration of BSO (15 mM) to produce an inhibition of cell growth 73%. These results points to an important involvement of this enzyme in the Sb^{III}-resistance phenotype of *L. guyanensis*.

4. Discussion

Trypanosomatids present a unique thiol-based redox system that depends on a low molecular mass thiol, trypanothione, absent in mammals and indispensable for parasite survival, hence, being a promising target for chemotherapy. Besides of its central role in

Table 1
Effective concentration (EC_{50}) of Sb^{III} of *L. guyanensis* lines in the absence or presence of DMFO or BSO.

Leishmmania lines	Sb^{III} EC_{50} (95% CI)						
	Sb^{III} susceptibility assay		Pharmacological inhibition tests				
	Sb^{III} (μM) 48 h	RI	Sb^{III} (μM) 24 h	Pre-treated 50 μM DFMO - 24 h	SI	Pre-treated 10 mM BSO - 24 h	SI
				Sb^{III} (μM) - 24 h		Sb^{III} (μM) - 24 h	
*Lg*WT	25.9 (24.45–27.51)	–	32.4 (30.53–34.4)	0.05 (0.0054–0.49)	648	1.6 (0.946–2.8)	20
*Lg*BSD	26.4 (25.08–27.86)	–	–	–	–	–	–
*Lg*ODC c10	58.7 (53.99–63.95)	2.2	78.4 (63.3–97.05)	50.9 (42.33–61.42)	1.5	–	–
*Lg*ODC c23	55.2 (52.16–58.44)	2.1	–	–	–	–	–
*Lg*GSH1 c3	87.7 (75.64–101.8)	3.4	99.3 (68.08–145)	–	–	24.06 (19.62–29.5)	4.1
*Lg*GSH1 c19	111.2 (92.9–133.1)	4.3	–	–	–	–	–

95% CI: Confidence Interval.
*Lg*WT: *L. guyanensis* wild-type.
*Lg*BSD: *L. guyanensis* transfected with pIR1-BSD empty vector.
*Lg*ODC: *L. guyanensis* transfected with pIR1-*Lg*ODC.
*Lg*GSH1: *L. guyanensis* transfected with pIR1-*Lg*GSH1.
c: clones.
RI: Resistance Index = Sb^{III} EC_{50} from *Lg*ODC or *Lg*GSH1 clones/Sb^{III} EC_{50} from *Lg*WT.
SI: Susceptibility Index = Sb^{III} EC_{50} from *Lg*WT, *Lg*ODC or *Lg*GSH1 clones without inhibitor/Sb^{III} EC_{50} from each line in the presence of the specific inhibitor.

antioxidant metabolism, trypanothione is a target of antimony since this compound has been shown to alter the parasite's intracellular redox homeostasis by depleting trypanothione and glutathione and by inhibiting trypanothione reductase (Wyllie et al., 2004). Moreover, several studies have demonstrated an association between high trypanothione levels and antimony-resistant in *Leishmania* spp. (Mukhopadhyay et al., 1996, 2011; Mandal et al., 2007), which was ascribed to the overexpression of enzymes from its biosynthetic pathway such as: ODC and GSH1. However the majority of these studies were carried out in *Leishmania* species from Old World. Thus, understanding the role of ODC and GSH1 enzymes in antimony-resistance in New World *Leishmania* species can help to identify new targets and design appropriate combination therapies for disease treatment.

Leishmania species are capable to produce *de novo* and obtain polyamines from the host (Jiang et al., 1999; Boitz et al., 2009). Ornithine decarboxylase, the rate limiting enzyme of the spermidine biosynthetic pathway, has been shown to play an important role in parasite growth and virulence. A *L. donovani* strain knockout for *odc* was unable to sustain an efficient *in vitro* growth and infection of macrophages or mice except that intracellular polyamine levels were restored in the parasite by genetic or metabolic (Boitz et al., 2009; Olenyik et al., 2011). The biological relevance of ODC for parasite survival *in vitro* and *in vivo* was further confirmed in these studies by pharmacological inhibition of the enzyme with DFMO, a specific and irreversible inhibitor of ODC (Bacchi et al., 1980; Kaur et al., 1986). Moreover, several studies reported a link between polyamine levels and drug resistance. For instance, transfection of *odc* in *L. donovani* increased the mRNA, protein and enzymatic activity levels of ODC, which yielded parasites resistant to antimony (Singh et al., 2007). The ODC mRNA levels, enzymatic activity, as well as putrescine and spermidine levels were increased in *in vitro* selected As^{III}-resistant *L. tarentolae* (Haimeur et al., 1999). However, Sb^{III}-resistant *L. tarentolae* showed no difference in ODC mRNA levels when compared to the parental cell line (Haimeur et al., 2000). *L. donovani* field isolates resistant to sodium antimony gluconate (SAG) presented gene amplification and an increased content and activity of ODC, as well as of putrescine and spermidine (Mukherjee et al., 2007; Singh et al., 2007; Rai et al., 2013). On the other hand, isolates of *L. donovani* resistant to Sb^{V} from Nepal presented lower ODC expression level compared to a sensitive isolate (Decuypere et al., 2005). Altogether, these data show differences in the expression and regulation of *odc* gene among antimony-resistant *Leishmania* species isolated from field or

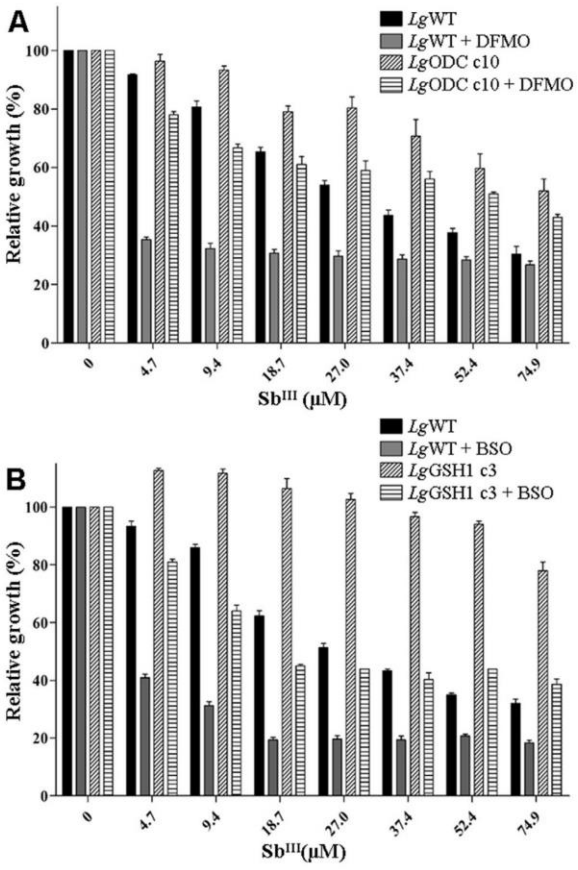

Fig. 3. Susceptibility to Sb^{III} of *L. (V) guyanensis* cell lines treated with a specific inhibitor of (A) ODC (DFMO) and (B) GSH1 (BSO). (A) *Lg*WT and *Lg*ODC c10 parasites were pre-incubated with 50 μM DFMO (EC_{50} for *Lg*WT) for 24 h (B) *Lg*WT and *Lg*GSH1 c3 were pre-treated with 10 mM BSO (EC_{50} for *Lg*WT) for 24 h. Subsequently the parasites were platted and incubated additional 24 h with different Sb^{III} concentrations (4.7–74.9 μM). Cells were counted using a Z1 Coulter Counter and the relative cell growth with respect to non-treated parasites was estimated. The values shown represent the mean and standard deviations from three independent experiments. *Lg*WT: wild-type, *Lg*ODC c10: clone 10 from ODC overexpressor, *Lg*GSH1 c3: clone 3 from GSH1 overexpressor.

obtained by *in vitro* selection.

As shown here, overexpression of ODC in the New World species *Leishmania guyanensis* conferred a higher resistant towards DFMO and Sb^{III}, added alone or in combination. The degree of resistance to Sb^{III} of the transgenic cell line was proportional to its ODC content, where against the combination DFMO/Sb^{III} the ODC-overexpressor displayed a 600-fold lower susceptibility than wild-type parasites. At this point it is important to recall that DFMO is an irreversible inhibitor and that the half-life of *L. mexicana* ODC (76% protein sequence identity with *Lg*ODC) was estimated in >6 h (Carrillo et al., 2000). Thus, the striking difference in sensitive towards DFMO/Sb^{III} between both cell lines can be ascribed to the fact that under the assay conditions (DFMO added at the EC_{50} for wild-type cells, 50 μM, for 24 h) a higher fraction of the endogenous ODC remains inhibited in the parental cell line with respect to that expressed by the transgenic cell line. Studies carried out with several *L. (V.) panamensis* laboratory- or field-strains resistant to Sb^{V} revealed that except for Sb^{V}-resistant amastigotes, which presented a 21% lower antimony-resistance upon treatment with DFMO, the sensitivity towards Sb^{III} of most strains was not enhanced in the presence of high DFMO concentrations (6 mM (Goyeneche-Patino et al., 2008)). Probably *L. panamensis*, at variance with *L. guyanensis*, have efficient mechanisms to import exogenous polyamines (Basselin et al., 2000) and, thus, succeed in circumventing the effects of ODC inhibition.

γ-Glutamylcysteine synthetase is the first and rate limiting step enzyme from the glutathione biosynthetic pathway that in related *Trypanosoma* species has been shown to exert kinetic control on the trypanothione pool (Lueder and Phillips, 1996; Olin-Sandoval et al., 2012). GSH1 has been shown to be essential for *L. infantum*, where it confers protection against oxidative stress and Sb^{V} (Mukherjee et al., 2009). Several reports have identified *gsh1* as a marker of resistance to (Arana et al., 1998; Mukherjee et al., 2007; Goyeneche-Patino et al., 2008; Moreira et al., 2013; Rai et al., 2013; Singh et al., 2014) and to therapeutic failure (Torres et al., 2010) in different pathogenic *Leishmania* species. For the non-pathogenic *L. tarentolae*, the co-amplification of a region containing the *mrpa* and *gsh1* genes was observed in As^{III}– and Sb^{III}-resistant cells, which was associated with high levels of their transcripts, glutathione and trypanothione (Grondin et al., 1997; Haimeur et al., 2000; Guimond et al., 2003). However, the overexpression of *gsh1* alone or in combination with *mrpa* did not confer *L. tarentolae* with resistance to Sb^{III} but, only the latter, to As^{III} (Grondin et al., 1997; Haimeur et al., 2000), suggesting the participation of additional molecules in the resistant mechanism towards these drugs. Different Sb^{III}/As^{III}-resistant *Leishmania* species reduced their resistance index to these drugs when treated with BSO (3–5 mM) (Grondin et al., 1997; Arana et al., 1998; Goyeneche-Patino et al., 2008). Here we show that overexpression or pharmacological inhibition of GSH1 in *L. guyanensis* de- or sensitize, respectively, the parasites against the detrimental effect of Sb^{III}.

The mode of action of Sb^{III} in *Leishmania* parasites is not completely understood, but it has been shown that the drug alters the mitochondrial membrane potential, leading to an increase in ROS production with concomitant fragmentation of DNA (Mehta and Shaha, 2006). On the other hand, Sb^{III} forms conjugates with glutathione and/or trypanothione, which are extruded from the cell by membrane pumps or sequestered into vesicles (Rai et al., 2013), hence contributing to deplete the pool of free thiols required to counteract the cytotoxic effect of ROS. Although our data indicate that ODC and GSH1 protects against the cytotoxic effects of antimony, the degree of contribution of each protein differs significantly. For instance, the susceptibility index of WT cells towards Sb^{III} was two orders of magnitude higher in parasites treated with DFMO than in those exposed to BSO. While ODC inhibition should only deplete trypanothione but not glutathione, the inhibition of GSH1 should lower the content of both thiols. A possible explanation for such discrepancy between this hypothetical metabolic scenario and our susceptibility data lies on a protective effect exerted by free polyamines. Cumulative evidence shows that polyamines are able to scavenge ROS and present a high affinity to bind, in particular spermidine, at varied sites of the DNA, where they not only regulate gene expression (e.g. stress-related genes) but also provide protection against ROS damage *in vitro* and *in vivo* (Bryson and Greenall, 2000; Rhee et al., 2007). Thus, it is tempting to speculate that upon ODC inhibition, the concomitant depletion of polyamines and trypanothione, will render the WT cells far more sensitive to Sb^{III} than those with a low thiol content. Future studies determining the polyamines or trypanothione levels of overexpressors lines and analyzing the reversion of growth inhibition by adding exogenous products of the GSH1 and ODC enzymes activities using theirs specific inhibitors will address all these issues and dissect the contribution to the antimony-resistance phenotype of other components from the parasite redox-system.

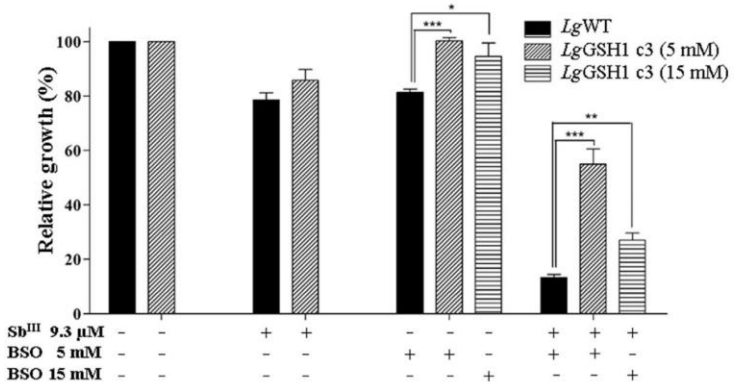

Fig. 4. Effect of BSO on the growth of *L. (V.) guyanensis* wild-type and GSH1-overexpressor treated with a sublethal concentration of Sb^{III}. Parasites (2×10^6 cells mL^{-1}) were seeded into 24-well plates containing M199 medium. Parasites were exposed or not to SbIII (9.3 μM) alone or with 5 mM (for *Lg*WT and *Lg*GSH1 c3) or 15 mM (only for *Lg*GSH1 c3) BSO. The incubation was extended for 48 h and parasite number was assessed using a Z1 Coulter Counter. The values represent mean and standard deviations from three independent experiments. Statistically different values (Student *t*-test, GraphPad Prism 5.0) are indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.0001$.

In conclusion, our data showed the direct involvement of ODC and GSH1 in Sb^{III}-resistance in *L. guyanensis* and provided evidence that a combined therapy with inhibitors of polyamine and/or glutathione/trypanothione biosynthesis may be a valuable strategy to minimize emergence of drug resistance, reduce drug toxicity and increase Sb^{III}-treatment efficacy for the disease caused by this parasite species.

Conflict of interest

The authors have declared no conflict of interest.

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