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

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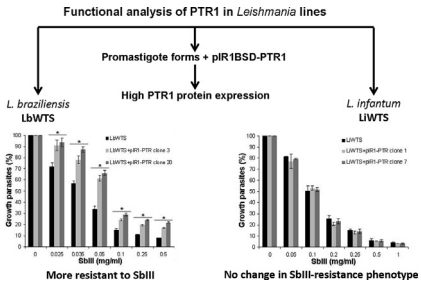
HIGHLIGHTS

- *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.

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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 x 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

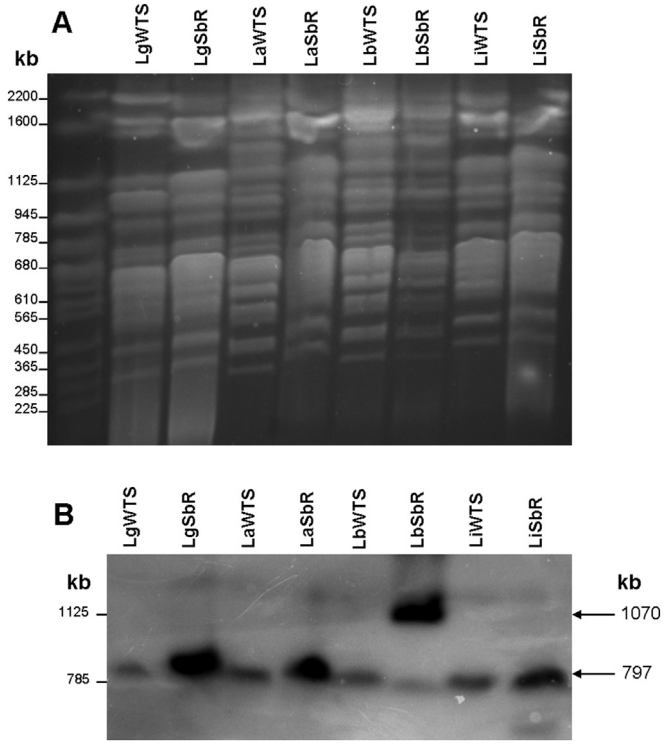

Fig. 1. Chromosomal location of *ptr1* gene in wild-type (WTS) and SbIII-resistant (SbR) *Leishmania* lines. (A) Chromosomal bands from the *Leishmania* lines were separated by PFGE and stained with ethidium bromide. (B) Chromosomal band profiles hybridized with a ^{32}P-labeled *ptr1*-specific probe.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

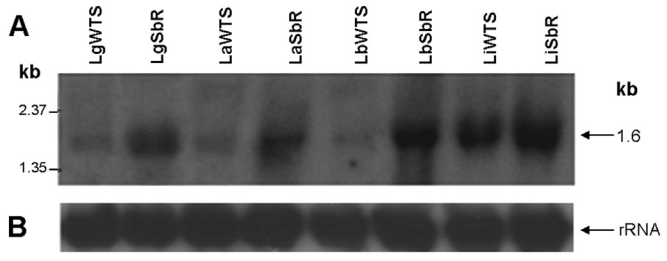

Fig. 2. Northern blot analysis of the *ptr1* gene from wild-type (WTS) and SbIII-resistant (SbR) *Leishmania* lines. (A) Northern blot profiles of total RNA extracts obtained using a ^{32}P-labeled *ptr1*-specific probe. (B) As quantitative control, the membrane was exposed to a *Leishmania 24S ribosomal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

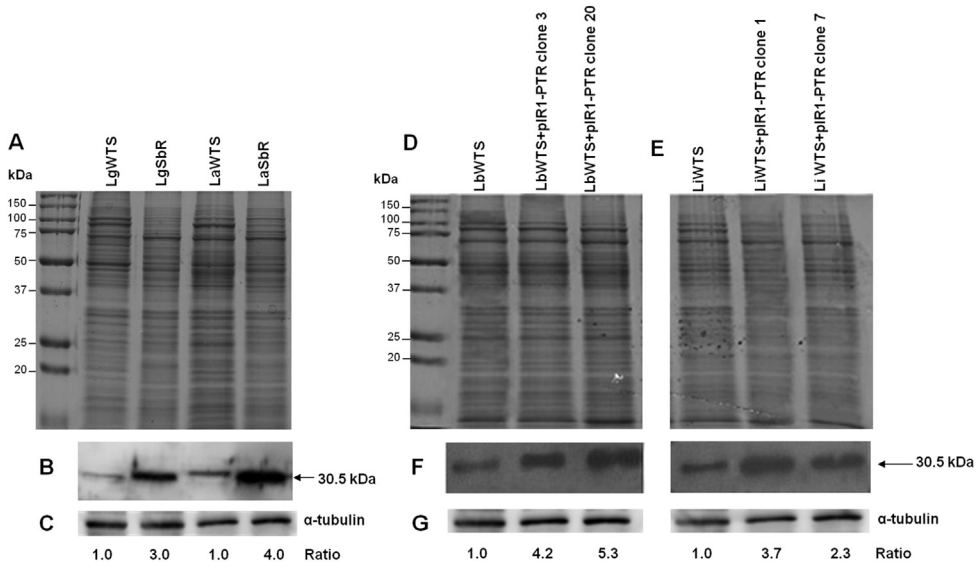

Fig. 3. Levels of PTR1 protein expression in wild-type (WTS) and SbIII-resistant (SbR) *L. guyanensis* and *L. amazonensis* lines, and in clonal lines from wild-type *L. braziliensis* and *L. infantum* non-transfected or transfected with the constructs pIR1BSD-PTR1. Proteins (40 μg) were separated on 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.

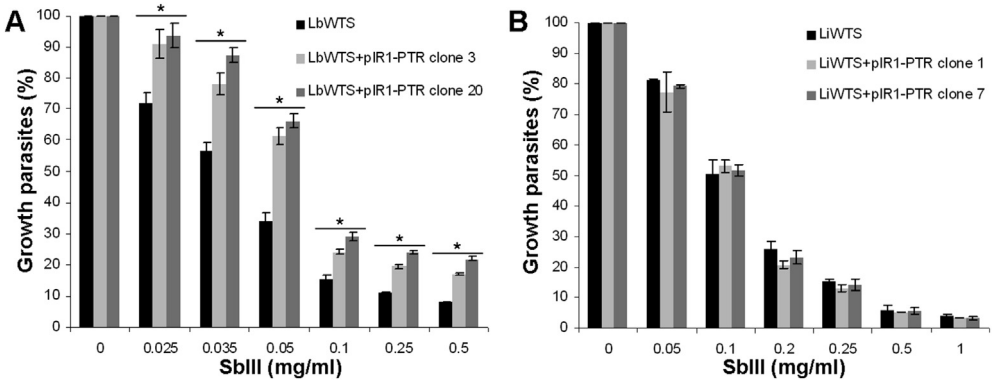

Fig. 4. Susceptibility assay to SbIII of clonal lines from wild-type (WTS) *L. braziliensis* (A) and *L. infantum* (B) lines non-transfected or transfected with the construct pIR1BSD-PTR1. Parasites were incubated in the absence or presence of different concentrations of SbIII (0.025–1.0 mg/ml) for 48 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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