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Comparative proteomic analysis of antimony-resistant and -susceptible *Leishmania braziliensis* and *Leishmania infantum chagasi* lines

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ABSTRACT

The emergence of drug-resistant *Leishmania* species is a significant problem in several countries. A comparative proteomic analysis of antimony-susceptible and antimony-resistant *Leishmania braziliensis* (LbSbR) and *Leishmania infantum chagasi* (LcSbR) lines was carried out using two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry (LC/MS/MS) for protein identification. Out of 132 protein spots exclusive or up-regulated submitted to MS, we identified 80 proteins that corresponded to 57 distinct proteins. Comparative analysis of data showed that most of the protein spots with differential abundance in both species are involved in antioxidant defense, general stress response, glucose and amino acid metabolism, and cytoskeleton organization. Five proteins were commonly more abundant in both SbIII-resistant *Leishmania* lines: tryparedoxin peroxidase, alpha-tubulin, HSP70, HSP83, and HSP60. Analysis of the protein abundance by Western blotting assays confirmed our proteomic data. These assays revealed that cyclophilin-A is less expressed in both LbSbR and LcSbR lines. On the other hand, the expression of pteridine reductase is higher in the LbSbR line, whereas tryparedoxin peroxidase is overexpressed in both LbSbR and LcSbR lines. Together, these results show that the mechanism of antimony-resistance in *Leishmania* spp. is complex and multifactorial.

1. Introduction

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*, and this is one among the six major tropical diseases in developing countries according to the World Health Organization (WHO). It is estimated that 12 million people are infected and 350 million are at risk of infection [1]. Leishmaniasis includes a spectrum of diseases with clinical and pathological characteristics represented by visceral (VL), cutaneous (CL), and mucocutaneous forms (MCL) [2]. The difficulties in controlling the vectors, in eliminating domestic reservoirs and in obtaining an accurate diagnosis of patients has led to an increased occurrence of deaths [1,3].

Pentavalent antimonials (SbV) are being used as first line drugs in the treatment of all leishmaniasis forms in South America, North Africa, Turkey, Bangladesh, and Nepal. Despite its use for treatment of leishmaniasis for over 70 years, the mechanism of action of antimony is still unclear. Studies suggest that SbV inhibits macromolecular biosynthesis in amastigotes [4], possibly altering energy metabolism by inhibiting glycolysis and fatty acid oxidation [5]. Other studies show that trivalent antimony (SbIII) causes disturbances in the thiol redox potential, which would lead to parasite death [6].

The emergence of antimony-resistant *Leishmania* species is a relevant problem in several countries. The resistance to this class of drugs has reached epidemic proportions in Bihar (India), where more than 60% of patients with visceral leishmaniasis were unresponsive to SbV treatment [7,8]. Even though the mechanism of antimony-resistance in *Leishmania* spp. has been widely studied,

Abbreviations: SbIII, potassium antimonyl tartrate; LbWTS, *L. (V.) braziliensis* Wild-type; LbSbR, *L. (V.) braziliensis* SbIII-resistant; LcWTS, *L. (L.) infantum chagasi* Wild-type; LcSbR, *L. (L.) infantum chagasi* SbIII-resistant; 2-DE, two-dimensional gel electrophoresis; MS, mass spectrometry.

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many questions remain unanswered [9]. SbV is a prodrug that must be reduced to SbIII in order to exert antileishmanial activity. Decreased reduction to SbIII was demonstrated in amastigotes of *Leishmania donovani* that were resistant to pentavalent antimonials [10]. Increased levels of trypanothione (TSH) were also found in SbIII-resistant parasites [11]. The exposure of parasites to SbIII causes efflux of TSH and inhibition of trypanothione reductase, thereby perturbing the thiol redox potential [6].

Since gene regulation in trypanosomatids is predominantly post-transcriptional, a proteomic approach has been used extensively to study differential protein expression in various *Leishmania* species [12,13]. In addition, the genome sequencing of different *Leishmania* species, such as *Leishmania infantum* and *Leishmania braziliensis* [14], has complemented the proteomic studies. In the last decade, many studies have been conducted using proteomic analysis of *Leishmania* spp. lines. They has contributed to the understanding of the molecular changes in the different stages of the life cycle of these parasites [15–18], as well as to the identification of vaccine candidates [19,20] and new drug targets [21].

Several groups have used the proteomic approach for understanding the mechanisms of drug resistance in *Leishmania* spp. This includes resistance to methotrexate in *Leishmania major* lines [21], arsenite in *L. donovani* samples [22], and gentamicin in *L. infantum* lines [23]. With regard to antimony-resistant parasites, proteomic analysis was performed using samples of *L. donovani* from clinical isolates resistant to SbV [24–26] and SbIII-resistant *L. infantum* [27] and *Leishmania panamensis* [28] lines. The results revealed that the resistant parasites exhibited changes in the levels of proteins associated with key metabolic pathways and in those involved in general stress responses and detoxification.

Several antimony resistance mechanisms in *Leishmania* spp. have been described in the literature. However, many questions remain unanswered, partly due to the interspecific variability of the parasite. As the vast majority of studies in this field have used Old World *Leishmania* species, little is known about the mechanisms of drug resistance in the New World species. Recently, we have in vitro selected *L. braziliensis* and *L. infantum chagasi* lines that are 20 and 4-fold more resistant to potassium antimony tartrate (SbIII) than their susceptible counterparts, respectively [29]. In the New World, *L. infantum chagasi* is the causative agent of VL, whereas *L. braziliensis* causes CL and MCL [30,31]. These species are extremely relevant to public health in Latin America. Therefore, we designed the present study to perform a comparative proteomic analysis of SbIII-resistant and -susceptible *L. braziliensis* and *L. infantum chagasi* lines by using two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry analysis (MS) for protein identification. We have also investigated the abundance levels of some differentially expressed proteins, such as pteridine reductase, cyclophilin, and tryparedoxin peroxidase in the SbIII-resistant and -susceptible *L. braziliensis* and *L. infantum chagasi* lines by using 1-DE and 2-DE Western blotting assays.

2. Materials and methods

2.1. Leishmania spp. samples

Promastigote forms of *L. braziliensis* (MHOM/BR/75/M2904) and *L. infantum chagasi* (MHOM/BR/74/PP75) were used in this study. Parasites were grown at 27 °C in M-199 medium according to [29]. All experiments were performed with parasites in the logarithmic phase of growth. The SbIII-resistant lines, named *L. braziliensis* resistant (LbSbR) and *L. infantum chagasi* resistant (LcSbR), were previously obtained from *L. braziliensis* wild-type (LbWTS) and *L. infantum chagasi* wild-type (LcWTS) by continuous stepwise drug pressure with SbIII [29]. Pairs of SbIII-susceptible and SbIII-resistant samples were cultivated under identical conditions. For each pair of parasites, three independent cultures were obtained and the parasites were snap frozen in liquid nitrogen and stored at −70 °C for protein extract preparations.

2.2. Protein extraction

Protein extracts were obtained by direct lysis of parasites in lysis buffer [8 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 20 mM Tris base and an half of protease inhibitor cocktail tablet *Complete Mini Protease Inhibitor Cocktail Tablets* (Roche) for each 10 ml of lysis buffer] in a proportion of 100 μl for 3.5×10^8 promastigotes. After homogenization for 2 h at room temperature (RT), the parasite homogenates were passed 10 times through a syringe (needle 30-gauge) and centrifuged at 20,000 × *g* for 30 min, at 25 °C. The supernatant containing total protein extract was stored at −70 °C until use. Protein concentration was quantified by the Bradford method.

2.3. Two-dimensional gel electrophoresis (2-DE)

Leishmania total proteins from each triplicate sample of *L. braziliensis* (500 μg) and of *L. infantum chagasi* (700 μg) were solubilized in IEF rehydration buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 0.0025% bromophenol blue, 0.2% (v/v) Bio-lyte (pH 3–10, Bio-Rad) and 65 mM DTT to a final volume of 350 μl. In the first dimension, proteins were applied to the immobilized pH gradient (IPG) strips 17 cm, pH 3–10 nonlinear or pH 4–7 (Bio-Rad) by in-gel sample rehydration. Isoelectric focusing was carried out using a Protean IEF Cell (Bio-Rad) at 20 °C and a 50 μA/strip. Passive rehydration was performed for 4 h, followed by an active rehydration at 50 V for 12 h. Isoelectric focusing was increased gradually to 8000 V and run for 40,000 V-h. The IPG strips were equilibrated for 15 min at RT under agitation in a reducing equilibration buffer containing 6 M urea, 30% (v/v) glycerol, 2% SDS, 50 mM Tris–HCl pH 8.8, 0.001% bromophenol blue, and 130 mM DTT, and for an additional 15 min in the alkylating equilibration buffer containing 135 mM iodoacetamide instead of DTT. The second dimension electrophoretic protein separation was performed using 12% SDD-PAGE in a Protean II XL Multi-Cell (Bio-Rad) at 16 °C. Electrophoresis was carried out under 50 V constant voltage for the first hour and then under 200 V, until the dye front reached the bottom of the gel. For visualization of the protein spots, the gels were stained with Colloidal Coomassie Blue G-250 [32].

2.4. Gel imaging and analysis

The gels were scanned under visible light using a GS-800 calibrated densitometer (Bio-Rad). Comparative analysis of digitized proteome images was performed using the image analysis software PDQuest 8.0 2D (Bio-Rad). Triplicate proteome images for SbIII-resistant and -susceptible *L. braziliensis* or *L. infantum chagasi* lines were aligned and matched to generate a composite map image. The intensity of each protein spot was normalized relative to the total abundance of all valid spots. After normalization and background subtraction, the triplicate proteome images for each pair of samples, LbWTS and LbSbR, LcWTS and LcSbR, were aligned and the matched set were created. The authenticity of each spot was validated by visual inspection and edited when necessary. Differences between normalized spot densities with Student's *t*-test values of $p < 0.05$ were considered significant when average triplicate images were compared. Significant differences in the densities (at least 2-fold) of protein spots between SbIII-susceptible and -resistant parasite proteomes were identified by pairwise comparisons.

2.5. Protein identification

The spots selected as differentially abundant (at least 2-fold) between SbIII-resistant and SbIII-susceptible *L. braziliensis* or *L. infantum chagasi* lines, and also those detectable only in one line, were manually excised directly from the replicate Colloidal Coomassie Blue-SDS PAGE gels and destained in 50% (v/v) acetonitrile (Fisher Scientific)/25 mM NH_4HCO_3 (Sigma) pH 8.0 until clear of blue stain. The gel fragments were dehydrated in 100% acetonitrile and completely dried in a SpeedVac (Eppendorf). In gel trypsin digestion was performed using 20 μg/mL of modified porcine trypsin (Sequencing grade, Promega, Madison, WI) in 25 mM NH_4HCO_3 pH 8.0 at 37 °C for 24 h. Tryptic peptides were extracted using 5% (v/v) formic acid (Merck)/50% (v/v) acetonitrile. Peptides were then concentrated in a SpeedVac to about 10 μl and stored at −20 °C. Subsequently, 40 μL 25 mM NH_4HCO_3 pH 8.0 were added to peptides and then concentrated in a SpeedVac to about 10 μL for mass spectrometry analysis at the Laboratory of Proteomics, Center of Biotechnology and Genetics – Universidade Estadual de Santa Cruz – UESC, Ilheús (Bahia, Brazil). Peptide MS/MS spectra were obtained by liquid chromatography coupled to electrospray tandem mass spectrometry (LC–ES-MS/MS) according protocol described by [33]. The sequences of peptides were searched against databases Swissprot (http://expasy.org/tools/tagdent.html) and NCBInr (http://blast.ncbi.nlm.nih.gov/). In addition, the resulting MS/MS spectra were submitted to database analysis using the MASCOT software (Matrix Science, London, UK). The search parameters allowed two tryptic missed cleavages, monoisotopic masses with unrestricted protein molecular weight and 0.3 Da and 0.1 Da mass tolerance of peptide and fragment, respectively. Partial carboxamidomethylation of cysteine and oxidation of methionine were considered in the search. A protein was considered a good identification if at least one peptide was confidently matched with database sequences, and an MASCOT score above 51 for individual peptides were considered significant for identity ($p < 0.05$ – peptide that reached probability greater than 95%). In order to avoid false positive protein identification, during the search, every time a protein sequence from the target database was tested, decoy sequences of the same length were automatically generated and tested using the Decoy function as a search parameter in the MASCOT software. No false positive hits were observed. When matches were obtained to predicted hypothetical proteins, the protein sequence was submitted to BLAST analysis to investigate a putative function. Gene Ontology (GO) annotations (biological process) for *L. braziliensis* and *L. infantum* proteins were assigned according to those reported in the GeneDB database (http://www.genedb.org). Also the results were confirmed at the KEGG Orthology (KO) database (http://www.genome.jp/kegg/kegg2.html).

2.6. Western blotting

Protein extracts (20 μg) obtained as above were loaded on 7 cm non-linear IPG strips pH 3–10 or IPG strips pH 5–8 (Bio-Rad) and submitted for isoelectric focusing using Protean IEF Cell (Bio-Rad) at 20 °C and 50 μA/strip. Passive rehydration was performed for 4 h, followed by an active rehydration at 50 V for 12 h. Isoelectric focusing was increased gradually to 4000 V and run for 16,000 V-h. Subsequently, the second dimension electrophoretic protein separation was performed in 12% or 15% SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated for 16 h at 4 °C with rabbit polyclonal antibodies specific for each protein of interest: *L. major* anti-pteridine reductase (1:100) (kindly provided by Dr. Stephen Beverley, Washington University, USA), *T. cruzi* antitryparedoxin reductase (1:500) [34] and *T. cruzi* anti-cyclophilin-A (1:5000) (kindly provided by Dr. Jaqueline Bua, Universidad de Buenos Aires, Argentina). The blots were washed and then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG 1:2000 (GE Healthcare). The membranes were washed, incubated with ECL Plus chemiluminescent substrate (GE Healthcare) and exposed to X-ray film. To confirm equivalent loading, SDS-PAGE containing the same samples were stained with Colloidal Coomassie Blue G-250 [32].

Table 1
Analysis of 2-DE protein profiles from SbIII-resistant and -susceptible *L. braziliensis* and *L. infantum chagasi* lines.

Leishmania lines	Spots/gel	Spots		MS/MS[c]	ID[d]
		Exclusive (unique)[a]	Up-regulated[b]		
LbWTS	360	46	20	26	20
LbSbR		10	43	39	21
LcWTS	320	2	11	13	09
LcSbR		27	33	54	30
Total		85	107	132	80

[a] Number of spots only detected in one of the samples of the pair SbIII-resistant (SbR) or susceptible (WTS).
[b] Number of spots detectable in both samples of the pair but more abundant in one of them (significant difference in the intensity, at least 2-fold, Student's *t*-test $p < 0.05$).
[c] Number of spots analyzed by MS/MS, mass spectrometry.
[d] ID, number of identified proteins.

3. Results and discussion

Total proteins of log phase promastigotes of LbWTS and LbSbR, LcWTS and LcSbR were initially loaded on 17 cm non-linear IPG strips pH 3–10. However, results showed that most spots were concentrated in the region between pH 4 and 7 (data not shown). Because of this result, IPG strips pH 4–7 were selected for constructing the 2-DE map of *L. braziliensis* and *L. infantum chagasi* lines (Fig. 1). Image analysis was performed using PDQuestTM software for three 2-DE gels obtained from three independent biological replicates. Protein spot profiles in 2-DE gels obtained from these preparations were highly reproducible in relation to the total number, localization, and density of the spots (Fig. 1S and 2S – Supplementary Material).

The comparative analysis of the 2-DE gel images showed significant difference of proteome profiles between the two *Leishmania* species analyzed, belonging to different subgenera (Fig. 1A and B). The number of protein spots analyzed per pair of *Leishmania* samples (Susceptible/Resistant) was 320 protein spots for *L. infantum chagasi* and 360 for *L. braziliensis*. Table 1 shows the number of total, exclusive and up-regulated protein spots in each sample and the number of spots that were submitted to MS/MS analysis and identified in each group. Of 192 spots exclusive (unique) and up-regulated in the *Leishmania* samples analyzed, 132 spots were submitted to MS/MS analysis for protein identification, since they were well-defined and reproducibly detected in the 2-DE gels. Among these, 80 were identified as 57 distinct proteins. These proteins were grouped in classes (Tables 2 and 3

) according to the biological process annotations (Gene Ontology and KEGG Orthology databases).

3.1. Biological process of proteins identified

3.1.1. Protein folding/chaperones and stress proteins

Heat shock proteins protect cells against external stimuli that cause cell damage. HSP70 and HSP83 proteins are highly evolutionarily conserved and are associated with chemical and physical stresses and they also play vital roles in normal cell function [35,36]. In our study, we observed that spots corresponding to HSP70 and

Table 2
Protein spots with differential abundance in SbIII-resistant (LbSbR) and SbIII-susceptible (LbWTS) *L. braziliensis* lines.

Spot no.	Ratio[b] LbSbR/LbWT	Protein identity	TriTrypDB accession no. *L. braziliensis*	M_r [c]	pI[d]	MS/MS MASCOT score[e]	% coverage/ matches[f]	Ref.[g]
Protein folding/chaperones and stress proteins[a]								
1	2.6	Heat shock protein 83-1	LbrM33.0340	80,532/44.96	5.04/4.97	669	21/20 (4)	[24,25]
2	2.5	Chaperonin Hsp60, mitochondrial precursor	LbrM35.2250	59,382/33.91	5.5/4.46	460	20/8 (5)	[26]
3	2.0	Heat shock protein 70-related protein	LbrM26.1260	70,879/47.02	6.65/-	365	20/12 (2)	[24–26,28]
Cytoskeletal proteins								
4	U	Alpha-tubulin	LbrM13.0190	49,684/43.48	4.65/5.65	562	35/17 (5)	[26,28]
5	U	Alpha-tubulin	LbrM13.0190	49,684/18.17	4.65/5.19	118	7/3 (1)	[26,28]
6	2.2	Alpha-tubulin	LbrM29.2700	28,689/15.74	4.89/4.75	346	11/9 (3)	[26,28]
7	U	Beta-tubulin	LbrM33.0920	49,745/30.26	4.45/5.95	184	10/5 (2)	[25–27]
8	2.1	Beta-tubulin	LbrM33.0920	49,745/37.42	4.45/4.92	652	30/9 (2)	[25–27]
Antioxidant/detoxificantion								
9	U	Tryparedoxin peroxidase	LbrM15.1100	22,572/21.80	6.9/5.36	63	4/1 (1)	
10	5.0	Tryparedoxin peroxidase	LbrM15.1100	22,572/23.23	6.9/5.46	350	47/14 (2)	
11	4.2	Tryparedoxin peroxidase	LbrM15.1100	22,572/22.08	6.9/5.43	100	11/2 (1)	
12	3.7	Tryparedoxin peroxidase	LbrM15.1100	22,572/23.22	6.9/5.42	300	34/10 (3)	
13	2.1	Tryparedoxin peroxidase	LbrM15.1100	22,572/21.91	6.9/5.74	151	19/5 (2)	
14	U	Pteridine reductase 1	LbrM23.0300	30,689/31.08	5.56/5.8	382	31/5 (4)	
15	2.4	3-hydroxyacyl-ACP dehydratase, MaoC, putative	LbrM07.0450	27,646/17.45	5.14/4.90	339	45/9 (5)	
RNA/DNA processing								
16	2.7	Proliferative cell nuclear antigen (PCNA), putative	LbrM15.1440	32,821/39.37	4.83/4.66	319	36/7 (3)	[25]
Protein biosynthesis								
17	U	Seryl-tRNA synthetase	LbrM19.0220	53,671/27.34	5.29/5.6	313	13/6 (3)	
18	U	Small ubiquitin protein, putative (SUMO)	LbrM08.0500	12,361/17.21	4.98/4.67	99	20/2 (1)	
Metabolic enzymes								
Glycolysis								
19	2.0	Enolase	LbrM14.1330	46,743/52.41	5.48/5.40	221	16/5 (1)	[24,25]
TCA cycle and Oxidative phosphorylation								
20	2.2	Reiske iron-sulfur protein precursor, putative	LbrM34.1450	34,045/27.54	6.02/5.30	305	24/11 (3)	
21	2.1	ATPase beta subunit, putative	LbrM25.1150	56,398/57.26	5.19/4.86	963	48/26 (12)	[28]

Spot no.	Ratio[b] LbWTS/LbSbR	Protein identity	TriTrypDB accession no. *L. braziliensis*	M_r [c]	pI[d]	MS/MS MASCOT score[e]	% coverage/ matches[f]	Ref.[g]
Protein folding/chaperones and stress proteins[a]								
22	7.8	Glucose-regulated protein 78, putative	LbrM28.1300	71,780/66.73	5.18/5.22	1217	36/22 (14)	
23	3.5	Cyclophilin a	LbrM25.0790	18,562/19.10	8.21/-	409	63/13 (6)	
Cytoskeletal proteins								
24	U	Actin-like protein, putative	LbrM15.1280	6165/50.93	5.4/5.30	229	20/6 (1)	
25	4.0	Paraflagellar rod protein 1D	LbrM31.0160	69,131/71.00	5.36/5.30	559	21/12 (7)	
26	3.7	Beta-tubulin	LbrM33.0920	49,745/32.00	4.65/4.70	1485	42/53 (25)	[25–27]
RNA/DNA processing								
27	2.9	RNA-binding protein, putative	LbrM34.2130	30,283/34.51	7.85/6.46	557	39/14 (7)	[26]
Protein biosynthesis								
28	2.2	Elongation factor 1-alpha	LbrM17.0090	48,941/39.07	8.49/-	680	35/25 (5)	[26]
29	2.0	Nascent polypeptide associated complex subunit-like protein, copy 1	LbrM04.0750	18,196/24.25	4.45/4.45	181	40/5 (1)	[26]
Metabolic enzymes								
TCA cycle and Oxidative phosphorylation								
30	U	ATPase beta subunit, putative	LbrM25.1150	56,267/27.45	5.19/4.98	291	20/6 (4)	[28]
31	2.2	Vacuolar ATP synthase subunit B, putative	LbrM28.2630	55,400/55.80	5.72/5.92	554	38/13 (5)	

Table 2 (*Continued*)

Spot no.	Ratio[b] LbWTS/LbSbR	Protein identity	TriTrypDB accession no. *L. braziliensis*	M_r [c]	p*I*[d]	MS/MS MASCOT score[e]	% coverage/ matches[f]	Ref.[g]
Amino acid metabolism and proteolysis								
32	U	Aminoacylase, N-acyl-L-amino acid amidohydrolase, putative	LbrM20.5930	43,449/31.87	5.49/5.14	247	22/7 (1)	
33	U	Nitrilase, putative	LbrM26.2200	31,381/32.95	6.45/5.70	340	37/7 (4)	
34	2.8	Glutamine synthetase, putative	LbrM06.0350	42,220/45.33	5.71/6.00	432	32/14 (6)	
35	2.6	Cathepsin L-like protease	LbrM08.0830	47,976/26.97	6.81/4.35	134	19/3 (1)	
Dephosphorylation								
36	3.0	Protein phosphatase, putative	LbrM25.0630	45,867/47.04	4.71/4.58	809	43/19 (8)	[26]
Unknown biological process								
37	2.6	Surface antigen protein, putative	LbrM12.0750	96,449/26.97	4.69/4.27	222	18/7 (3)	
Hypothetical								
38	U	Hypothetical protein, conserved	LbrM09.0120	65,475/66.31	5.32/5.31	634	31/14 (5)	
39	U	Hypothetical protein, conserved	LbrM23.1530	27,271/27.50	6.6/5.71	408	33/9 (4)	
40	U	Hypothetical protein, conserved	LbrM26.2570	43,957/48.83	5.49/5.13	203	8/3 (2)	
41	2.2	Hypothetical protein, conserved	LbrM35.6250	41,601/44.82	4.88/4.71	942	36/27 (14)	

[a] Biological process annotations according to Gene Ontology in GeneDB and KEGG Orthology databases.

[b] Ratio: relative abundance of protein spots in SbR and WTS lines were determined by densitometry using PDquest Program. The mean optical density of each protein spot was determined from triplicate gels of each pair, and then converted to a ratio of more abundance SbR/WTS or WTS/SbR.

[c] M_r, molecular weight – predicted (Da)/experimental (kDa).

[d] p*I*, isoelectric point – theoretical/experimental.

[e] Global MASCOT scores. In this study a protein was considered a good identification if at least one peptide was confidently matched with database sequences, and an MASCOT score above 51 for individual peptides were considered significant for identity ($p < 0.05$ – peptide that reached probability greater than 95%).

[f] % coverage/matches – sequence covered percentage/number of peptides matched (number in brackets indicate the number of peptides that reached probability greater than 95% – Table 1S supplementary material). Tandem mass spectra were searched against the NCBI nonredundant database using the MASCOT software (http://www.matrixscience.com).

[g] Ref., proteins also identified by others proteomic analysis of Sb-resistant *Leishmania* spp. lines; U unique, means protein spot only detected in one of the samples of the pair SbIII-resistant or SbIII-susceptible. p*I* (–) indicate that the p*I* value was not determined in pH 4–7 IPG strips.

HSP83 proteins were more abundant in both LcSbR and LbSbR lines (Tables 2 and 3). In the LcSbR line, a mitochondrial and cytoplasmic HSPs70 were approximately 2 to 3-fold more abundant than that in the susceptible line, LcWTS. In *L. braziliensis*, one protein spot identified as HSP70 was 2-fold more abundant in the LbSbR line compared to the susceptible line, LbWTS. Regarding HSP83, the results showed that two protein spots identified were 2.4 and 2.6-fold more abundant in LcSbR and LbSbR lines respectively than their susceptible pairs.

Overexpression of heat shock protein variants of different molecular weights has been observed in arsenite-resistant [37] and in antimony-resistant [24–26,28,38] *Leishmania* spp. lines. HSP83 and HSP70 are also involved in the activation of programmed cell death mediated by drugs, as they interfere with the mitochondrial membrane potential of *L. donovani* [24,25]. Data suggest that expression of HSP70 and HSP83 is a primary nonspecific stress response of parasite. However, this response is critical to allow cells to develop more efficient and specific antimony resistance mechanisms [21,39,40].

The spot corresponding to another heat shock protein, the mitochondrial precursor of chaperonin HSP60, was more abundant 2.5 and 2.8-fold in LcSbR and LbSbR lines, respectively. HSP60 responds to heat shock and is mainly found in mitochondria and chloroplasts [41]. The spot corresponding to stress-induced protein STI1, a co-chaperone produced in response to stress, which forms a complex with heat shock proteins HSP70 and HSP83, was 6-fold less abundant in the LcSbR line. In this study, we also observed that protein spots corresponding to glucose-regulated protein-78, GRP-78, a member of the HSP70 family, and cyclophilin-A, a molecular chaperone, were 7.8 and 3.5-fold less abundant in the LbSbR line, respectively. Cyclophilin-A possesses a peptidylprolyl cis/trans isomerase activity and is a key molecule in many biological functions, including molecular chaperoning, protein folding, protein trafficking, immune modulation, and signal transduction [42].

3.1.2. Cytoskeletal proteins

Our data identified three protein spots detectable only in LbSbR line corresponding to alpha and beta-tubulin cytoskeletal proteins. Other two spots identified as these same proteins were 2-fold more abundant in the LbSbR line. In the SbIII-resistant *L. infantum chagasi* line, two protein spots corresponding to alpha-tubulin variants and one to actin were only detectable or more abundant compared to the paired susceptible line. In agreement with our results, previous reports have shown that the tubulins were also more abundant in arsenite-resistant *L. donovani* samples [43]. These authors also reported changes in the expression levels and dynamics of tubulin polymerization and in the regulation of its distribution, as well as induction of apoptosis as components of the *L. donovani* response to arsenite [44]. Increased expression of tubulin was also observed in the cytosolic fraction of SbV-resistant *L. donovani* isolates [25,26], in axenic amastigotes of SbIII-resistant *L. infantum* lines [27], in methotrexate-resistant *L. major* [21], in SbIII-resistant *L. panamensis* [28], and gentamicin-resistant *L. infantum* [23]. In this study, we also observed that protein spot identified as beta-tubulin and paraflagellar rod protein 1D were found 3.7 and 4-fold less abundant in LbSbR line than in its susceptible pair LbWTS,

Table 3
Protein spots with differential abundance in SbIII-resistant (LcSbR) and SbIII-susceptible (LcWTS) *L. infantum chagasi* lines.

Spot no.	Ratio[b] LcSbR/LcWT	Protein identity	TriTrypDB accession no. *L. infantum*	M_r [c]	pI[d]	MS/MS MASCOT score[e]	% coverage/ matches[f]	Ref.[g]
Protein folding/chaperones and stress proteins[a]								
42	3.0	Heat-shock protein hsp70, putative	LinJ28.3060	80,730/24.27	5.73/5.48	294	9/6 (2)	[24–26,28]
43	2.2	Heat shock protein, putative	LinJ18.1350	92,644/70.00	5.24/5.20	87	4/2 (1)	
44	2.1	Heat shock 70-related protein 1, mitochondrial precursor, putative	LinJ30.2530	72,119/71.49	5.7/5.62	708	26/16 (6)	[24,26]
45	2.8	Chaperonin HSP60, mitochondrial precursor	LinJ36.2140	59,663/59.83	5.33/4.94	341	15/6 (4)	[26]
46	2.4	Heat shock protein 83-1	LinJ33.0350	79,249/84.32	5.14/4.87	547	17/12 (3)	[24,25]
Cytoskeletal proteins								
47	U	Actin	LinJ04.1250	42,307/25.21	5.41/5.54	104	11/2 (1)	
48	U	Alpha-tubulin	LinJ13.1450	36,933/12.03	6.78/5.53	164	11/3 (1)	[26,28]
49	2.9	Alpha-tubulin	LinJ13.1450	36,933/12.03	6.78/5.53	81	6/2 (1)	[26,28]
Antioxidant/detoxificantion								
50	3.5	Peroxiredoxin, putative/tryparedoxin peroxidase	LinJ23.0050	25,582/18.14	6.43/5.320	160	27/4 (1)	
51	2.5	Peroxiredoxin, putative/tryparedoxin peroxidase	LinJ23.0050	25,582/17.44	6.43/5.72	352	34/13 (5)	
Protein biosynthesis								
52	2.6	Elongation factor 2	LinJ36.0200	94,942/50.56	5.77/5.83	290	7/5 (2)	[26,28]
Metabolic enzymes								
Glycolysis								
53	U	Pyruvate dehydrogenase E1 beta subunit, putative	LinJ25.1790	38,448/37.77	5.64/5.54	250	28/8 (2)	
TCA cycle and oxidative phosphorylation								
54	U	Inorganic pyrophosphatase, putative	LinJ03.0890	25,320/26.37	5.15/5.82	112	22/2 (1)	
55	U	Isocitrate dehydrogenase, putative	LinJ33.2680	46,583/48.51	5.43/5.56	118	12/3 (1)	
Amino acid metabolism and proteolysis								
56	U	S-adenosylmethionine synthetase	LinJ30.3560	43,498/57.19	5.5/5.88	255	14/5 (3)	[28]
57	3.0	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase, putative	LinJ25.2090	30,723/23.20	5.8/5.65	107	10/3(1)	
58	2.1	Aminopeptidase P1 (Metallo-peptidase, Clan MG, Family M24), putative	LinJ02.0010	69,409/71.45	5.6/6.14	273	9/5 (3)	[26]
Dephosphorylation								
59	U	Protein phosphatase 2C-like protein	LinJ36.0560	33,460/28.90	5.2/5.53	97	10/2 (1)	
60	2.7	Protein phosphatase, putative	LinJ25.0780	45,795/49.97	4.75/4.78	458	29/20 (6)	[26]
Metabolism of cofactors and vitamins								
61	2.2	Biotin/lipoate protein ligase-like protein	LinJ31.1070	28,946/25.82	6.07/6.78	138	16/3 (2)	
Fatty acid metabolism								
62	2.2	3-hydroxyisobutyryl-coenzyme A hydrolase-like protein	LinJ32.3810	39,541/45.77	5.48/5.76	59	13/3 (1)	
Mediated signal transduction								
63	2.0	Rab7 GTP binding protein, putative	LinJ18.0890	24,298/20.26	5.27/5.59	57	4/1 (1)	[28]
Hypothetical								
64	U	Hypothetical protein, conserved	LinJ36.6170	41,313/48.48	5.17/5.26	214	19/5 (3)	
65	U	Hypothetical protein, conserved	LinJ32.0660	21,190/17.07	5.61/5.88	142	19/4 (1)	
66	U	Hypothetical protein, conserved	LinJ35.1220	24,796/20.14	6.43/6.83	99	27/3 (1)	
67	6.0	Hypothetical protein, conserved	LinJ34.2410	22,036/12.84	9.59/6.29	61	14/2 (1)	
68	2.8	Hypothetical protein, conserved	LinJ27.1920	22,432/20.76	4.73/4.77	160	11/2 (1)	
69	2.2	Hypothetical protein, conserved	LinJ35.4540	21,687/21.00	4.15/4.15	99	11/2 (1)	
70	2.0	Hypothetical protein, conserved	LinJ26.1960	89,762/90.90	4.99/4.36	144	6/3 (1)	
71	2.0	Hypothetical protein, conserved	LinJ34.2530	28,783/35.09	5.43/5.45	57	5/1 (1)	

Table 3 (*Continued*)

Spot no.	Ratio[b] LcWTS/LcSbR	Protein identity	TriTrypDB accession no. *L. infantum*	M_r [c]	p*I*[d]	MS/MS MASCOT score[e]	% coverage/ matches[f]	Ref.[g]
Protein folding/chaperones and stress proteins[a]								
72	6.0	Stress-induced protein sti1	LinJ08.1020	62,770/71.07	5.9/6.47	706	29/15 (3)	[28]
73	2.2	Heat shock protein 83-1	LinJ33.0360	81,013/84.81	5.08/4.62	777	24/14 (4)	[24,25]
74	2.0	Heat shock 70-related protein 1, mitochondrial precursor, Putative	LinJ30.2480	72,089/72.20	5.8/5.81	1448	39/27 (8)	[24–26,28]
RNA/DNA processing								
75	2.0	RNA helicase, putative	LinJ21.1820	59,217/55.76	8.82/6.36	418	17/8 (4)	
Protein biosynthesis								
76	2.0	Elongation factor 1-alpha	LinJ17.0090	55,768/62.18	9.51/5.89	148	11/5 (1)	[26]
Metabolic enzymes								
Glycolysis								
77	3.1	Enolase	LinJ14.1240	46,634/61.98	5.33/5.77	1247	65/50 (22)	[24,25]
TCA cycle and oxidative phosphorylation								
78	U	Reiske iron-sulfur protein precursor, putative	LinJ35.1540	34,046/33.43	5.91/5.69	251	30/7 (2)	
79	2.2	Succinyl-CoA ligase, putative [GDP-forming] beta-chain	LinJ36.3100	44,498/48.23	6.77/6.65	353	24/8 (2)	
Amino acid metabolism and proteolysis								
80	4.2	Trypanothione reductase	LinJ05.0350	53,620/66.80	5.85/6.48	317	30/8 (1)	

[a] Biological process annotations according to Gene Ontology in GeneDB and KEGG Orthology databases.

[b] Ratio: relative abundance of protein spots in SbR and WTS lines were determined by densitometry using PDquest Program. The mean optical density of each protein spot was determined from triplicate gels of each pair, and then converted to a ratio of more abundance SbR/WTS or WTS/SbR.

[c] M_r, molecular weight – predicted (Da)/experimental (kDa).

[d] p*I*, isoelectric point – theoretical/experimental.

[e] Global MASCOT scores. In this study a protein was considered a good identification if at least one peptide was confidently matched with database sequences, and an MASCOT score above 51 for individual peptides were considered significant for identity ($p < 0.05$ – peptide that reached probability greater than 95%).

[f] % coverage/matches – sequence covered percentage/number of peptides matched (Number in brackets indicate the number of peptides that reached probability greater than 95% – Table 1S supplementary material). Tandem mass spectra were searched against the NCBI nonredundant database using the MASCOT software (http://www.matrixscience.com).

[g] Ref., proteins also identified by others proteomic analysis of Sb-resistant *Leishmania* spp. lines; U unique, means protein spot only detected in one of the samples of the pair SbIII-resistant or SbIII-susceptible.

respectively. On the other hand, actin-like protein was only detectable in LbSbR line.

3.1.3. Antioxidant/detoxification proteins

Interestingly, our results showed that pteridine reductase (PTR1) (LbrM23.0300) was present in one exclusive spot identified in the LbSbR line (Table 2). This enzyme is an NADPH-dependent reductase that participates in the salvage of pterins, converting biopterin to tetrahydrobiopterin, which are essential components for the growth of *Leishmania* [45,46]. A study conducted in *L. major* lines showed that this enzyme contributes to oxidative stress defense within the macrophage, suggesting that the mechanism of action of antimonials might be related to the production of reactive oxygen species [46]. Overexpression of PTR-1 was also observed in the methotrexate-resistant *L. major* lines [21].

Trypanosomatids present a unique mechanism for detoxification of peroxides that is dependent on trypanothione, in a process distinct from that found in vertebrates. Therefore, antioxidant defenses are a promising target for chemotherapy. Tryparedoxin peroxidase, a member of peroxiredoxin family, acts in antioxidant defense participating in an enzymatic cascade for the detoxification of hydroperoxides [47]. This enzyme is critical to the survival of *Leishmania* during oxidative stress generated by macrophages and by drugs [21]. In our study, four protein spots corresponding to tryparedoxin peroxidase were 2–5-fold more abundant in the LbSbR line than in its susceptible pair LbWTS (Table 2). In addition, one exclusive spot was also identified as tryparedoxin peroxidase in this line. These spots could represent different variants of tryparedoxin peroxidase, due to post-translational modifications, since a small difference between the calculated and experimental p*I* of identified spots were found. In addition, two protein spots corresponding to peroxiredoxin/tryparedoxin peroxidase were 2.5 and 3.5-fold more abundant in the LcSbR line than in the LcWTS (Table 3). It is important to note that high levels of tryparedoxin and tryparedoxin peroxidase were also observed in antimony-resistant *L. donovani* lines [48] and gentamicin-resistant *L. infantum* samples [23], which agrees with our findings. These results suggest that an increased metabolism of peroxides and higher antioxidant defense may play a significant role in the resistance of parasites to antimonials.

3.1.4. RNA/DNA processing and protein biosynthesis

In our study, one spot corresponding to proliferative cell nuclear antigen (PCNA) (LbrM15.1440), which participates in the processes of replication and DNA repair, was 2.7-fold more abundant in LbSbR line. In agreement with our findings, PCNA was also overexpressed in clinical isolates of *L. donovani* resistant to pentavalent antimony, suggesting that it probably plays a role in the mechanism of drug resistance [25]. Some spots corresponding to ribosomal proteins and proteins involved in the translation machinery were identified, such as elongation factor 2 (LinJ36.0200), which was 2.6-fold more abundant in the LcSbR line. Interestingly, elongation factor 2 was also more abundant in SbV-resistant *L. donovani* isolates [26] and SbIII-resistant *L. panamensis* lines [28]. Moreover, the protein spot elongation factor 1-alpha was reduced 2-fold in both SbIII-resistant *Leishmania* spp. lines analyzed here. This protein has been also implicated in other cellular processes such as signal transduction and apoptosis [49]. We have also identified protein spots detectable only in the LbSbR line corresponding to seryl-tRNA

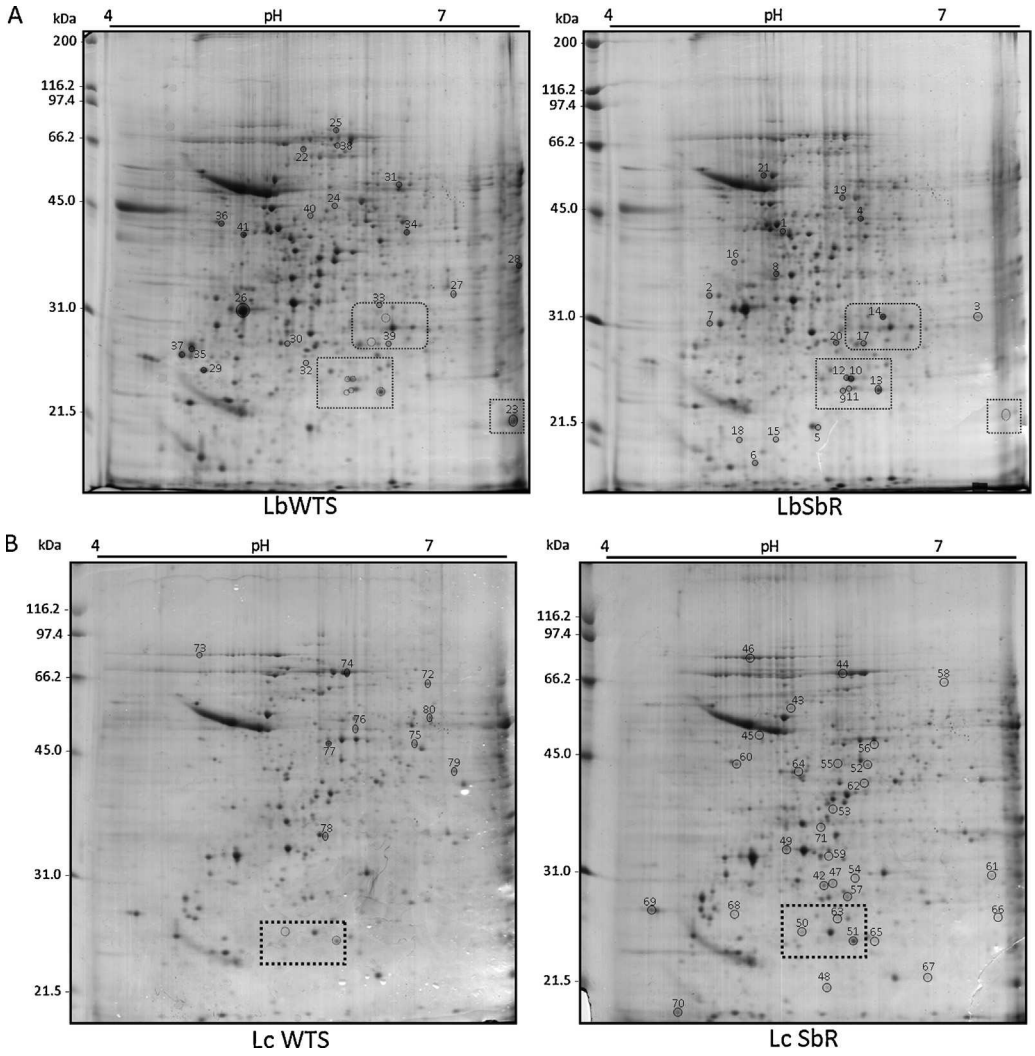

Fig. 1. 2-DE gels of proteins from susceptible (WTS) and SbIII-resistant (SbR) *L. braziliensis* (A) and *L. infantum chagasi* (B) lines. IEF was carried out with 500 μg (A) or 700 μg (B) of protein using 17 cm, 4–7 pH range IPG strips. SDS-PAGE was performed on 12% polyacrylamide gels and stained with Colloidal Coomassie Blue-G250. Inside circles indicate protein spots exclusively/differentially abundant per pair of *Leishmania* lines. The numbers refer to the spot identification used in Tables 2 and 3. The rectangles show different gel regions to be amplified (Figs. 3 and 4). Each gel is representative of three biological replicate.

synthetase (LbrM19.0220) and small ubiquitin protein (SUMO motif – *small ubiquitin-related modifier*). Protein modifications induced by SUMO have been implicated in the regulation of numerous biological processes, including transcription, protein localization, and cell cycle control [50]. Besides these proteins, one spot corresponding to nascent polypeptide associated complex subunit-like protein (LbrM04.0750) was 2-fold less abundant in the LbSbR line compared with its paired susceptible line, LbWTS.

3.1.5. Metabolic enzymes

Studies on the mechanism of action of pentavalent antimony (SbV) suggested that it inhibits biosynthesis of macromolecules, possibly via perturbation of energy metabolism, glycolysis, and fatty acid oxidation [40]. The promastigote forms of *Leishmania* depend mainly on the metabolism of glucose and amino acids for energy generation. In this study, protein spots corresponding to enzymes of the glycolytic pathway, tricarboxylic acid cycle, and oxidative phosphorylation were found to be differently abundant in both SbIII-resistant *Leishmania* spp. lines analyzed. A protein spot identified as pyruvate dehydrogenase E1 beta subunit (LinJ25.1790) was only detectable in LcSbR line. Enolase is involved in glycolysis and gluconeogenesis—the metabolic processes essential for *Leishmania* spp. [25,51]. The protein spot corresponding to this enzyme was reduced 3.1-fold in this LcSbR line and was increased 2-fold in the LbSbR line compared to their respective susceptible pairs. Enolase is also differentially abundant in SbV-resistant *L. donovani* isolates [24,25]. The spot of Reiske iron-sulfur protein precursor, involved in the metabolism of carbohydrates, proteins and lipids, was 2-fold more abundant in LbSbR line. On the other hand, one spot corresponding to this protein was only detectable in the LcWTS line. Spots corresponding to enzyme isocitrate dehydrogenase (LinJ33.2680) of the tricarboxylic acid cycle (TCA) and the inorganic pyrophosphatase (LinJ03.0890) of oxidative phosphorylation were only detectable in the LcSbR line compared with its susceptible pair LcWTS. Comparative quantitative proteomic analysis of clinical isolates of *L. donovani* that are susceptible and resistant to pentavalent antimony also revealed a significant increase in the expression of several glycolytic enzymes [26], corroborating our data. These data suggest that upregulation of these enzymes in antimony-resistant parasites favors enhanced glycolysis, which provides energy for their proliferation. In addition, high glycolysis rate helps reduce oxidative stress since pyruvate (a product of glycolysis) is a scavenger of peroxides [26].

Some proteins involved in amino acid metabolism and proteolysis presented differential abundance in the SbII-resistant *Leishmania* lines studied. We have observed that protein spots

corresponding to aminoacylase and nitrilase were only detectable in the LbWTS line. In addition, the protein spots glutamine synthetase and cathepsin-L were about 2.7-fold less abundant in LbSbR line than its susceptible pair LbWTS. We have observed that protein spots corresponding to aminopeptidase P1 and 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase, were 2 and 3-fold more abundant in the LcSbR line compared to its paired susceptible line, LcWTS. The regulation of these proteins may have a role in the survival of the parasite. Aminopeptidase [26] were also more abundant in SbV-resistant *L. donovani* isolates.

In *L. infantum chagasi*, we observed that the protein spot corresponding to *S*-adenosylmethionine synthetase (SAMS) was only detectable in the LcSbR line. SAMS enzyme plays a central role in the synthesis and assembly of trypanothione precursors and appears to be involved in the cellular detoxification process. SAMS also was more abundant in the SbIII-resistant *L. panamensis* lines [28], corroborating our data. As described in the literature, the overexpression of enzymes involved in maintaining thiol levels suggests a possible involvement these enzymes in response to drug exposure [28,38].

3.1.6. Hypothetical proteins

In our analysis, 12 protein spots that presented differential abundance were identified as hypothetical proteins, 8 in LcSbR and 4 in LbWTS. Of the protein spots identified as more abundant in the LcSbR line, one spot corresponding to a hypothetical protein containing structural domains compatible with HSP23 (LinJ35.4540), a co-chaperone for HSP90. In this same line, another spot was identified as chaperonin LINJ35.1220, which degrades unfolded proteins (*Degradation arginine-rich protein for mis-folding*-Armet). Besides these, we observed one hypothetical protein that has a domain Alba (LinJ34.2410), involved in the metabolism of RNA [52]. In agreement with our results, the expression of this protein is elevated in the gentamicin-resistant *L. infantum* line [23].

3.2. Analysis of the expression level of cyclophilin-A, pteridine reductase and tryparedoxin peroxidase in SbIII-resistant and -susceptible L. braziliensis and L. infantum chagasi lines

We assessed the expression level of cyclophilin-A (CyPA), pteridine reductase (PTR1), and tryparedoxin peroxidase (TRYP) in SbIII-resistant and SbIII-susceptible *L. braziliensis* and *L. infantum chagasi* lines by Western blotting assays. The spot protein corresponding to CyPA (LbrM.25.0790) was 3.5-fold less abundant in LbSbR compared to its paired susceptible line, LbWTS. To confirm this result, we determined CyPA expression level by 1-DE and 2-DE Western blotting assays using a polyclonal antibody, *T. cruzi* anti-cyclophilin-A (TcCyPA). This antibody was used because of the high identity between the CyPA amino acid sequences of *T. cruzi* compared to *L. braziliensis* and *L. infantum chagasi* (81% and 79%, respectively). Anti-TcCyPA antibody recognized two spots with the expected size of 19 kDa and p*I* 8.0 in the susceptible *L. braziliensis* line and one spot in the paired resistant line LbSbR (Fig. 2A), which coincides with that for CYPA (Fig. 1A). Regarding *L. infantum chagasi*, the anti-TcCyPA antibody recognized six spots in LcWTS and five spots in LcSbR, corresponding to the different isoforms of CyPA (LinJ.25.0940) with p*I*s ranging from 6.0 to 8.0, probably due to post-translational modifications of the protein (Fig. 2B). According literature data, cyclophilin-A of *L. infantum* has one site of acetylation justifying isoforms with p*I* more acidic [53]. It is important to mention that CyPA was not identified as differentially abundant in our comparative proteomic analysis between LcWTS and LcSbR since protein spots of low molecular weight were not analyzed due to not-well resolved zone at the end of the gel. Interestingly, we observed that in both *Leishmania* species studied, the expression of CyPA is lower in the SbIII-resistant lines compared to their SbIII-susceptible pairs. In the 1-DE Western blots, the antibody to TcCyPA recognized a 19 kDa band in all *Leishmania* samples studied (Fig. 3A–S Supplementary material). Densitometric analysis of the CyPA band using alpha-tubulin as a reference (Fig. 3D–S) showed that the level of expression of the CyPA protein was 1.7 and 2.5-fold lower in the resistant LcSbR and LbSbR lines compared to theirs respective susceptible counterparts LcWTS and LbWTS, confirming our proteome data.

Comparative proteomic analysis between LbWTS and LbSbR lines showed that pteridine reductase LbrM.23.0300 (PTR1) was identified in one exclusive spot in LbSbR line (Fig. 3A). To confirm this result, we carried out 2-DE Western blotting using a polyclonal antibody that recognized PTR1 of *L. major*. The identity between the PTR1 amino acid sequences of *L. major* compared to *L. braziliensis* and *L. infantum chagasi* was 73% and 90%, respectively. Results showed that this antibody strongly recognized a spot of approximately 31 kDa corresponding to PTR1, only in the LbSbR line, confirming the proteomic analysis data (Fig. 3B). Our analysis of both *L. infantum chagasi* lines showed that PTR1 was not among the differentially abundant protein spots. In addition, 2-DE Western blotting showed that the anti-PTR1 antibody did not recognize any protein spot in LcWTS and LcSbR lines (data not shown). On the other hand, 1-DE Western blotting showed that the anti-PTR1 antibody recognized a band of 31 kDa for both *Leishmania* species analyzed. However, the level of expression of PTR1 protein was similar between the *L. infantum chagasi* lines (Fig. 3B–S). Whereas for *L. braziliensis*, densitometric analysis showed that the PTR1 was 7-fold more expressed in LbSbR compared to its susceptible counterpart LbWTS (Fig. 3B–S).

Our proteomic results showed that five spots were identified as tryparedoxin peroxidase LbrM.15.1100 (TRYP) in the LbSbR line (Fig. 4A). 2-DE Western blotting assays using an antibody against cytosolic tryparedoxin peroxidase of *T. cruzi* [34] revealed one spot in LbWTS and three spots in LbSbR (Fig. 4B). Two of the spots (spots 12 and 10) in the LbSbR line coincided with those identified by MS/MS (Fig. 4A). However, one spot from each line was not identified by MS/MS probably because of lack of material. Thus, the data suggest that the proteins recognized by this antibody correspond to the cytosolic isoform of TRYP. It is important to note that the identity between the TRYP amino acid sequences of *T. cruzi* compared to *L. braziliensis* and *L. infantum chagasi* correspond to 69% and 72%, respectively. Western blotting analysis using *L. infantum chagasi* showed that the anti-TcTRYP antibody recognized three spots with the expected size of approximately 20 kDa and p*I* 6.0; this corresponds to tryparedoxin peroxidase in both LcWTS and LcSbR lines (Fig. 4D). Interestingly, we observed that two spots showed a higher abundance in the LcSbR line compared to the susceptible pair LcWTS (spots 50 and 51), while one spot was present at similar levels in each line (Fig. 4C). These results confirm our proteomic analysis. These enzymes are encoded by several genes and are paralogs, encoding very similar proteins that differ in predicted size and p*I* [23]. 1-DE Western blotting analysis, the anti-TcTRYP antibody recognized a 20 kDa band in all *Leishmania* lines studied (Fig. 3C–S). Densitometric analysis of the TRYP band showed that this protein is 1.6 and 3-fold more expressed in the both SbIII-resistant lines (LcSbR and LbSbR) compared to theirs respective susceptible counterparts (LcWTS and LbWTS), confirming our proteome data (Fig. 3C–S).

In our study, 57 distinct proteins with differential abundance between SbIII-susceptible and -resistant *L. braziliensis* and *L. infantum chagasi* lines were identified by proteomic analysis. Among these, 17 were also previously identified [24–28] and the other 40 proteins were observed only in our proteomic analysis of SbIII-resistant *Leishmania* spp. lines. We believe that some of them are associated with the general stress response and others may be associated with the mechanism of resistance of *Leishmania* spp. to

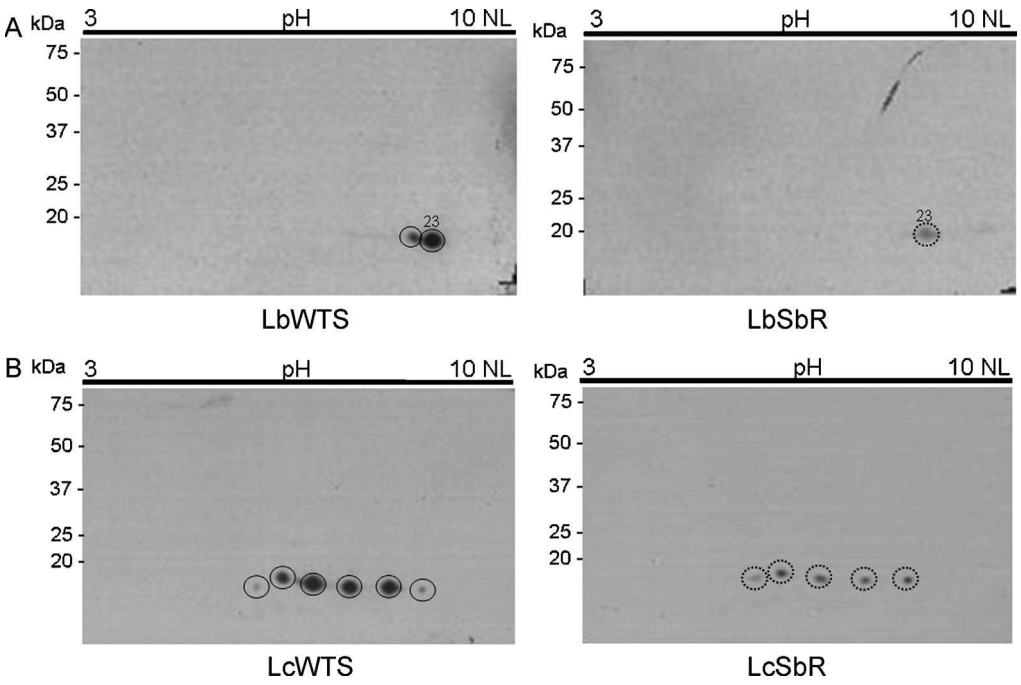

Fig. 2. 2-DE Western blotting analysis of CyPA expression in SbIII-susceptible and SbIII-resistant *Leishmania* spp. lines. Proteins (20 μg) from *L. braziliensis* (A) and *L. infantum chagasi* (B) were loaded on 7 cm non-linear IPG strips of pH 3–10, submitted to isoelectric focusing, separated on 15% SDS-PAGE and blotted onto nitrocellulose membranes. The blots were probed with a rabbit polyclonal antibody anti-cyclophilin-A of *T. cruzi* (1:1500) and developed using ECL. Protein spots with increased abundance are circled with full lines and those with decreased abundance with dotted lines.

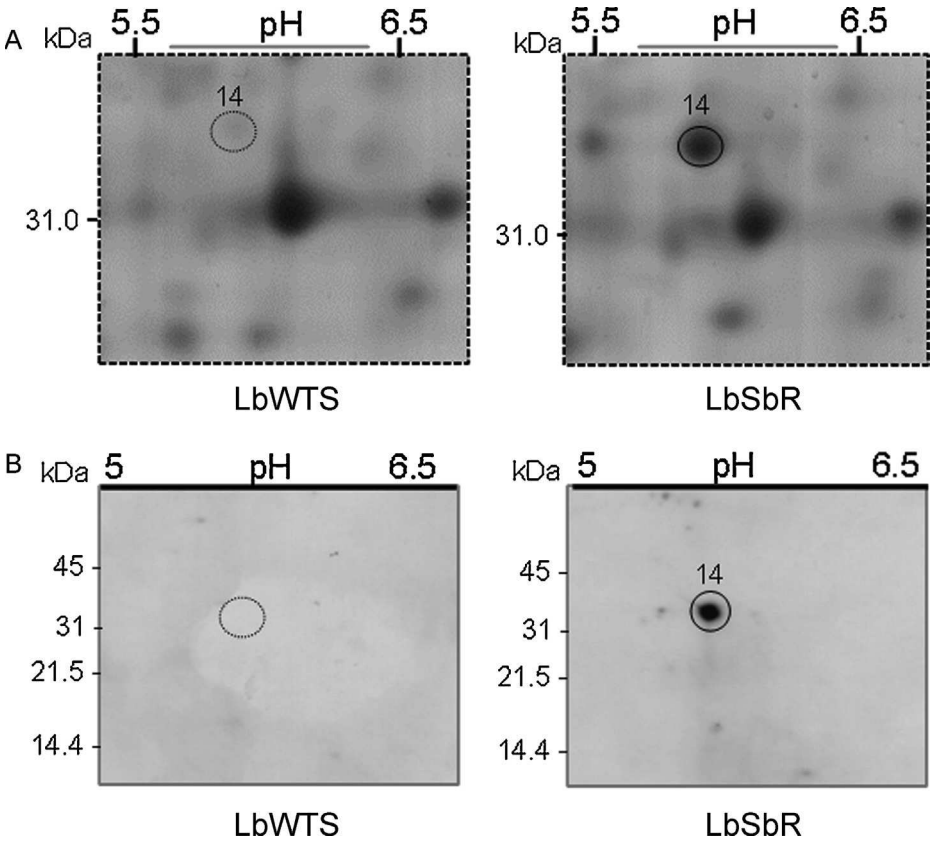

Fig. 3. Proteomic and 2-DE Western blotting analysis of PTR1 expression in *L. braziliensis* LbWTS and LbSbR lines. (A) Comparison of amplified regions of Colloidal Coomassie Blue-stained protein profiles of LbWTS and LbSbR from Fig. 1. One exclusive spot identified as PTR1 (14) by LC/MS/MS in the LbSbR is circled. (B) Proteins (20 μg) were loaded on 7 cm IPG strips of pH 5–8, submitted to isoelectric focusing, separated on 15% SDS-PAGE and blotted onto nitrocellulose membranes. The blots were probed with a rabbit polyclonal antibody anti-pteridin reductase of *L. major* (1:100) and developed using ECL. This antibody recognized one spot only in the LbSbR line.

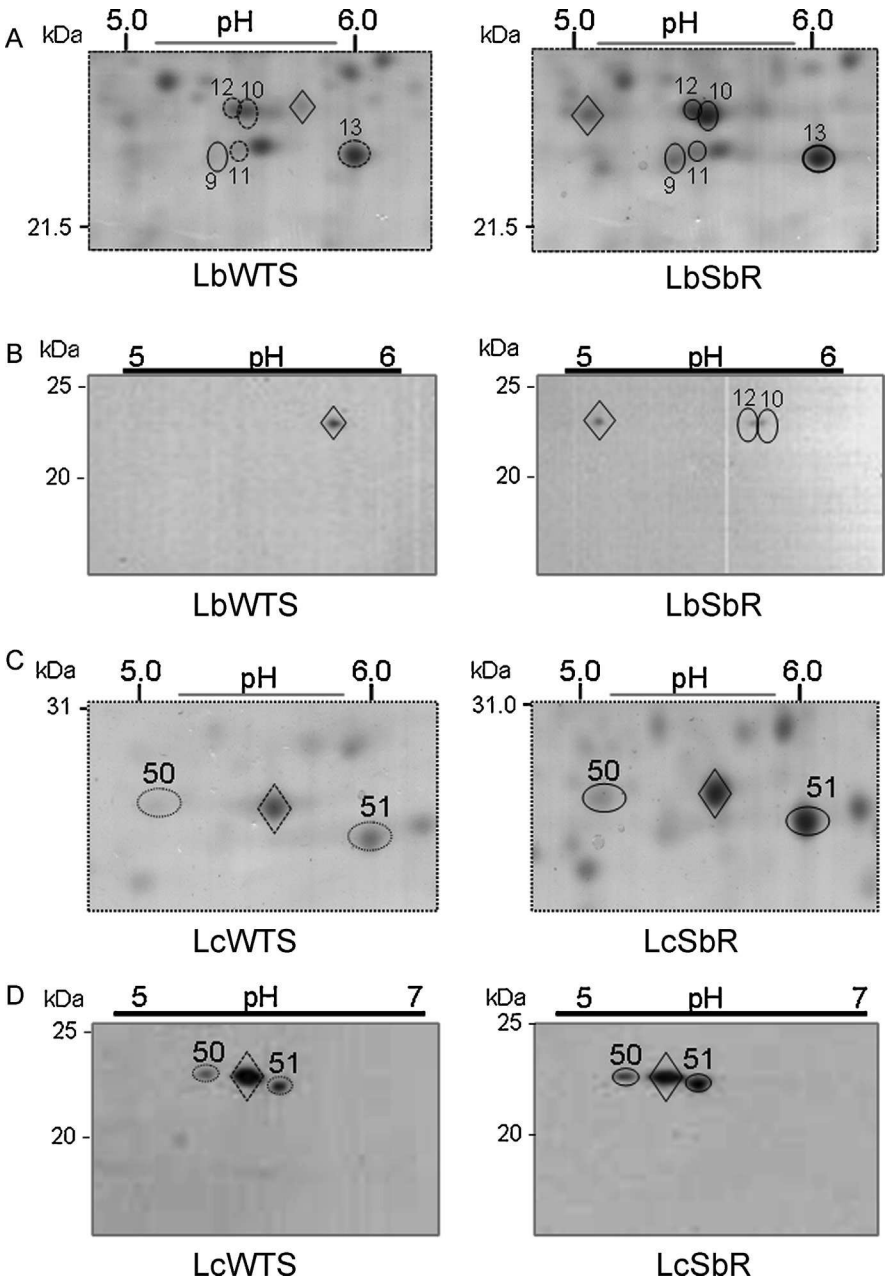

Fig. 4. Proteomic and 2-DE Western blotting analysis of tryparedoxin peroxidase expression in SbIII-susceptible and SbIII-resistant *Leishmania* spp. lines. Comparison of amplified regions of Colloidal Coomassie Blue-stained protein profiles of WTS and SbR *Leishmania* lines from Fig. 1 (IEF was carried out using 17 cm, 4–7 pH range IPG strips). Protein spots differentially abundant identified as TRYP by LC/MS/MS are circled in *L. braziliensis* – 5 spots: 9, 10, 11, 12 and 13 (A) and *L. infantum chagasi* – 2 spots: 50 and 51 (C). Proteins (20 μg) were loaded on 7 cm IPG strips of pH 5–8, submitted to isoelectric focusing, separated on 15% SDS-PAGE and blotted onto nitrocellulose membranes. The blots were probed with a rabbit polyclonal antibody anti-cytosolic tryparedoxin peroxidase of *T. cruzi* (1:1500) and developed using ECL. This antibody recognized one and three protein spots in LbWTS and LbSbR lines, respectively (B) and three protein spots in both *L. infantum chagasi* lines (D). Protein spots with increased abundance are circled with full lines and those with decreased abundance with dotted lines. The protein spots recognized by anti-TRYP antibody, but not identified by LC/MS/MS (B and D) are indicated by diamonds. The protein spots 9, 11 and 13 (A and B) were not recognized by anti-TRYP antibody.

antimony. Probably, the physiological changes of resistant parasites modify the flux of several pathways to elaborate a resistance phenotype. Ponte-Sucre [54] showed that the drug-resistance phenotype is also associated with changes in physiological events, such as parasite infectivity, incorporation of metabolites, xenobiotic and traffic conjugation, intracellular metabolism, cell morphology, and others. Increased abundance of enzymes involved in antioxidant defense is possibly a key feature of the antimony resistance mechanisms. In addition, a high rate of glycolysis reduces oxidative stress. On the other hand, the stress proteins such as cyclophilin-A and glucose-regulated 78 protein and enzymes of amino acid metabolism (aminoacylase, nitrilase, glutamine synthetase, and cathepsin) were less abundant in the resistant LbSbR line. Together, these results show that the mechanism of antimony resistance in *Leishmania* spp. is complex and multifactorial.

Several proteins that were differentially abundant in this study have been previously reported as potential therapeutic targets. We believe that understanding the role of the proteins differentially abundant in antimony-resistant *Leishmania* spp. lines presented in this study may increase our understanding of the drug-resistance

phenotype and the metabolism of these parasites, thus contributing to the development of new targets for chemotherapy of leishmaniasis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2013.06.006.

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