RESEARCH ARTICLE

Intrachromosomal Amplification, Locus Deletion and Point Mutation in the Aquaglyceroporin AQP1 Gene in Antimony Resistant Leishmania (Viannia) guyanensis

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Abstract

Background

Antimony resistance complicates the treatment of infections caused by the parasite Leishmania.

Methodology/Principal Findings

Using next generation sequencing, we sequenced the genome of four independent Leishmania guyanensis antimony-resistant (SbR) mutants and found different chromosomal alterations including aneuploidy, intrachromosomal gene amplification and gene deletion. A segment covering 30 genes on chromosome 19 was amplified intrachromosomally in three of the four mutants. The gene coding for the multidrug resistance associated protein involved in antimony resistance was also amplified in the four mutants, most likely through chromosomal translocation. All mutants also displayed a reduced accumulation of antimony mainly due to genomic alterations at the level of the subtelomeric region of chromosome 31 harboring the gene coding for the aquaglyceroporin 1 (LgAQP1). Resistance involved the loss of LgAQP1 through subtelomeric deletions in three mutants. Interestingly, the fourth mutant harbored a single G133D point mutation in LgAQP1 whose role in resistance was functionality confirmed through drug sensitivity and antimony accumulation assays. In contrast to the Leishmania subspecies that resort to extrachromosomal amplification, the Viannia strains studied here used intrachromosomal amplification and locus deletion.

Conclusions/Significance

This is the first report of a naturally occurred point mutation in AQP1 in antimony resistant parasites.
Author Summary

Drug resistance remains a major concern in leishmaniasis chemotherapy, a neglected tropical disease that causes 60,000 deaths around the world annually. To better understand the molecular mechanisms behind drug resistance, we selected *L. guyanensis* parasites resistant to antimony, the first-line drug against this disease in many countries. Through whole-genome sequencing we found variations in the copy number of chromosomes in addition to gene amplification and gene deletion events in antimony-resistant parasites. A marker previously related to antimony resistance, the gene coding for multidrug resistant protein A, was found to be amplified. Transport studies revealed a reduced antimony accumulation in resistant parasites that we correlated with the deletion of the gene coding for the aquaglyceroporin 1 (AQP1) responsible for antimony uptake in *Leishmania*. Additionally, a point mutation in *AQP1* was found to be associated with antimony resistance. These findings may contribute to the development of new chemotherapy strategies against leishmaniasis.

Introduction

Leishmaniasis defines a spectrum of infectious diseases caused by protozoan parasites belonging to the genus *Leishmania* that are transmitted to mammals via the bite of sandflies. Leishmaniasis are neglected tropical diseases that could potentially affect ~ 350 million people in 98 countries [1]. Clinical manifestations differ widely depending on the host immune response and the *Leishmania* species responsible for infection and vary from visceral leishmaniasis—VL to cutaneous leishmaniasis—CL [2]. The clinical manifestations of CL can further vary from localized ulcerative skin lesions to destructive mucosal inflammation (mucocutaneous leishmaniasis—MCL), the latter being mostly associated with infections caused by the *Viannia* subgenus in South America [3–6].

No vaccine is available against leishmaniasis and chemotherapy thus represents the main strategy for the treatment of all forms of the disease [7]. Despite the introduction of paromomycin [8], amphotericin B [9] and miltefosine [10] in the anti-*Leishmania* arsenal, pentavalent antimony (SbV)-derived compounds have been used for more than 65 years and are still the first-line of treatment against leishmaniasis in many countries [11]. Drug combinations, short therapeutic schemes and single drug doses are solutions currently debated to avoid drug resistance, one of the major drawbacks against leishmaniasis especially in the case of antimony [12]. Antimonial resistance has first emerged against VL in India [13] but cases of treatment failure involving species from the *Viannia* subgenus have since been reported in Brazil [14,15], Peru [16] and Colombia [17]. Drug susceptibility screenings also supported the notion that antimony resistant *L. (Viannia)* parasites can develop in the field [18,19].

Antimony is most active against *Leishmania* in its trivalent form (SbIII) which is produced through the reduction of pentavalent antimony (SbV) possibly within the macrophage hosts [20] but also within *Leishmania* [21,22]. SbIII is then passively transported into *Leishmania* cells through aquaglyceroporin 1 (AQP1), a porin also allowing the transport of water, glycerol, urea, dihydroxyacetone, methylglyoxal and polyols [23]. SbIII is indeed a chemical mimic of natural AQP1 substrates, having a similar conformation and charge as glycerol [24]. AQP1 plays an important role in volume regulation and osmotaxis in *Leishmania* [25] and its reduced expression is associated with SbIII resistance [26]. On the other hand, re-sensitization is achieved when AQP1 is overexpressed in resistant parasites deficient for AQP1 [26,27]. Targeted mutagenesis of *L. major* AQP1 demonstrated a role for residues Glu125 and Ala163 located at the extracellular loop in SbIII susceptibility [28].
While several molecular mechanisms leading to antimony resistance in Leishmania have been described, resistance remains only partly understood and most likely constitutes a multifactorial process [29]. Next generation sequencing has been used to produce several L. donovani genomes and revealed genomic alterations and plasticity that correlated with antimony resistance [30,31]. Gene amplification is also frequently observed in both laboratory-raised or field isolates resistant to antimony, in which circular or linear extrachromosomal DNA are formed by homologous recombination and annealing of direct or inverted repeated sequences, respectively [32]. A well-studied example of such amplification is the gene coding for the multidrug resistance associated protein A (MRPA) which is frequently amplified as part of circular amplicons originating from chromosome 23 in SbIII-resistant strains [33–35] and whose role in resistance involves the intravesicular sequestration of Sb-thiol conjugates in SbIII-resistant Leishmania [36].

Our understanding of drug resistance mechanisms come from the analysis of parasites belonging to Leishmania subgenus and little is known about the mechanisms leading to antimony resistance in the Viannia group, with the exception of few recent studies that highlighted previously observed alterations [37,38]. In this study, whole-genome sequencing was performed in laboratory-selected antimony resistant (SbR) Leishmania (Viannia) guyanensis mutants aiming at the dissection of molecular mechanisms of SbIII resistance in Leishmania (Viannia) parasites.

Methods

Leishmania cultures and in vitro selection of SbIII resistance

Leishmania (Viannia) guyanensis (MHOM/BR/1975/M4147) promastigotes were axenically maintained in minimum essential culture medium (α-MEM) (Gibco, Invitrogen, Grand Island, NY, USA) at pH 7.0 supplemented with 10% (v/v) heat inactivated fetal bovine serum (Wisent Inc., St-Jean-Baptiste, QC, CA), 100 mg mL−1 kanamycin, 50 mg mL−1 ampicillin, 2 mM L-glutamine, 5 mg mL−1 hemin, 5 mM biopterin, (Sigma-Aldrich, St Louis, MO, USA) and incubated at 25°C. Four L. guyanensis SbIII-resistant mutants (LgSbIII650.1 to LgSbIII650.4) were independently selected from WT L. guyanensis in 25 cm² flasks containing 5 mL of α-MEM in the presence of increasing SbIII concentrations. Potassium antimonyl tartrate (Sigma-Aldrich, St Louis, MO, USA) was used as the source of SbIII. The stepwise drug selection ranged from 80 μM up to 650 μM of SbIII. Last-level SbR mutants were grown in absence of drug pressure for 26 passages to revert resistance. In addition, two independent L. guyanensis SbIII-resistant mutants (LgSbIII.1/2013 and LgSbIII.2/2013) were selected by SbIII increments (resistant to 80, 160, 240, 325 or 650 μM SbIII) and maintained in culture. For drug susceptibility assay, 10⁶ parasites mL−1 in mid-log phase growth were seeded in 24-wells cell culture plates containing 1.5 mL of α-MEM, incubated under gentle agitation at 25°C during 72 h in presence or absence of several concentrations of drug. Growth was monitored daily by measuring absorbance at 600 nm to obtain the Sb sensitivity profile [39].

Next-generation sequencing

Sequencing libraries were produced from 50 ng of phenol-extracted/ethanol-precipitated genomic DNA by using the Nextera DNA sample preparation kit (Illumina Inc, San Diego, CA, USA) according to manufacturer instructions. Genome sequences were determined by Illumina HiSeq1000 101-nucleotides paired-end reads. Sequencing reads were aligned to a Leishmania (Viannia) braziliensis (MHOM/BR/1975/M2904) reference genome (TriTrypDB version 6.0) [40] using the software package Burrows-Wheeler Alignment [41]. The maximum number of mismatches was 4, the seed length was 32 and 2 mismatches were allowed within the seed. The
detection of single nucleotide polymorphisms was performed using SAMtools (version 0.1.18), bcftools (distributed with SAMtools) and vcfutils.pl (distributed with SAMtools) [42]. Putative SNPs detected by whole genome sequencing were verified by conventional PCR amplification and DNA sequencing. Sequencing data are available at the EMBL-EBI European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession number PRJEB6114 with samples ERS434587, ERS434588, ERS434589, ERS434590 and ERS434591 corresponding to *L. guyanensis* WT, LgSbIII650.1, LgSbIII650.2, LgSbIII650.3 and LgSbIII650.4, respectively.

**Real time RT-PCR**

First-strand cDNA was synthesized from 5 μg of total RNA using Oligo d(T)12–18 and Super-Script II H-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol. Equal amounts of cDNA were run in triplicate and amplified in 25 μL reactions containing 1 x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 100 nM forward and reverse primers and 100 ng of cDNA target. Reactions were carried out using a rotator thermocycler Rotor Gene (RG 3000, Corbett Research, San Francisco, USA). Mixtures were initially incubated at 95°C for 5 min and then cycled 30 times at 95°C, 60°C and 72°C for 15 s. No-template controls were used as recommended. Three technical and biological replicates were established for each reaction. The relative amount of PCR products generated from each primer set was determined based on the cycle threshold—Ct value and the amplification efficiencies. Data were analyzed using the comparative $2^{-\Delta\Delta C_t}$ method. Gene expression levels were normalized to constitutively expressed mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, *LbrM*30.2950). Primers for targeted genes and internal gene expression control *GAPDH* were designed using PrimerQuest (http://www.idtdna.com/Primerquest/Home/Index) and sequences are listed in S1 Table.

**PFGE and Southern blot**

Molecular karyotype was obtained from *L. guyanensis* WT and Sb-resistant mutants by separation of chromosomes by pulse field electrophoresis [43]. 10^8 mid-log phase parasites were embedded in low melting point agarose blocks, digested with proteinase K and electrophoresed in a contour clamped homogenous electric field apparatus (CHEF Mapper, Bio-Rad, Hercules, CA, USA). The blocks were mounted in 1% agarose gel and electrophoresed in 0.5x Tris-Bo-rate-EDTA running buffer at 5 V cm⁻¹ with 120° separation angle at 14°C during 30 h. A range of 150 to 1500 kb was applied for a wide chromosomal separation, resolving most of *Leishmania* chromosomes in a single molecular karyotype gel. *Saccharomyces cerevisiae* chromosomes were used as DNA size marker (Bio-Rad, Hercules, CA, USA). For Southern blots, genomic DNA was isolated using DNAzol reagent (Life Technologies, Carlsbad, CA, USA) following manufacturer’s instructions and digested with the *PstI* restriction enzyme (New England Biolabs Inc, Ipswich, MA, USA). Digested genomic DNA or PFGE-derived molecular karyotype were transferred by capillarity onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech, Sunnyvale, CA, USA) and cross-linked with UV light. The blots were hybridized with [$\alpha$-³²P]dCTP labeled DNA probes according to standard protocols [44]. Primers used for southern blot probes are listed in S1 Table. Densitometric quantification of southern blot-derived bands was performed using Image J version 1.48a.

**Cloning and transfection of LgAQP1 in Leishmania**

The gene LgAQP1 (GenBank accession numbers KJ623262 and KJ623263) was amplified from genomic DNA of *L. guyanensis* WT and LgSbIII650.4 using primers containing 5’ *XbaI* and 3’ *HindIII* restriction sites, followed by cloning in pGEM T-easy (Promega, Madison, WI, USA).
The WT AQP1 and its LgSbIII650.4 variant were subcloned into the pSP72αZEOα expression vector, a derivative of pSP72αNEOα [45] in which the gene neomycin phosphotransferase (NEO) was replaced by the bleomycin-binding protein gene (ZEO) conferring resistance to zeocin [46]. To validate the expression of episomal LgAQP1, a green fluorescent protein (GFP)-tagged construct was made using a PCR fusion-based strategy as previously described [47] using primers listed in S1 Table. The GFP gene was amplified using the pSP72αNEOαGFP vector as template. The LgAQP1-GFP fusions were cloned into pGEM T-easy and subcloned into the XbaI/HindIII sites of pSP72αZEOα, resulting in the pSP72αZEOαLgAQP1WTGFP or pSP72αZEOαLgAQP1(G133D)GFP constructs that were transfected by electroporation as previously reported [45]. Transfected parasites were preselected in the presence of 500 μg mL⁻¹ of Zeocin Selection Reagent (Life Technologies, Carlsbad, CA, USA) and after 24 h, selection of transfectants was carried out in presence of 1 mg mL⁻¹ of Zeocin Selection Reagent. Before transfection, all constructs were confirmed by DNA sequencing.

**Western blot**

Total and membrane protein fractions were extracted from *Leishmania* as previously described [48]. Briefly, parasites were centrifuged and washed three times with ice-cold Hepes-NaCl at 3000 rpm for 5 minutes. The pellet was resuspended in a lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM DTT) and homogenized by vortexing after addition of proteases inhibitors cocktail (1 mg mL⁻¹ leupeptin, 2 μg mL⁻¹ aprotinine, 5 mM EDTA). The lysate was then incubated on ice for 15 min, followed by three cycles of freeze (-80°C) and thaw (37°C). The supernatant was recovered after centrifugation at 15000 rpm for 30 min at 4°C. From there, supernatant containing membrane fractions was kept at -80°C. Proteins were then extracted from membranes fractions using solubilisation buffer (50 mM Tris-HCl pH 8, 150 mM NH₄Cl, 2 mM MgCl₂, CHAPS 1%) by incubation on ice for 30 min. 50 μg of proteins were run on 10% acrylamide gel and transferred electrically onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked overnight in PBS (1 X), Tween (0.1%), Milk (5%). Membranes were incubated overnight at 4°C with a GFP monoclonal antibody (Roche, Basel, Switzerland) and an α- tubulin monoclonal antibody (Life Technologies, Carlsbad, CA, USA) diluted 1:1000 in PBS-Tween-Milk solution. Membranes were then washed three times for 5 min in PBS-Tween and incubated 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Thermo Fisher Scientific Inc, Waltham, MA, USA) diluted 1:10000 in PBS-Tween-Milk solution. Membranes were washed again three times and incubated with Immobilon western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

**Antimony uptake assay**

Antimony accumulation measurements were carried out based on previous studies [49,50]. Briefly, 10⁸ mid-log phase *Leishmania* promastigotes were washed and resuspended in 1 mL of Hepes/NaCl/Glucose buffer (20 mM HEPES, 0.15 M NaCl, 10 mM glucose, pH 7.2) followed by incubation with 540 μM SbIII at 25°C as previously described [38]. One hour of Sb incubation was chosen to compare differences in Sb accumulation among the conditions evaluated. Drug accumulation was stopped by incubating cells on ice followed by three washes with ice-cold Hepes/NaCl/Glucose buffer. Parasites were centrifuged at 1800 g during 5 min at 4°C and the dried pellet was digested in 100 μL of 65% HNO₃ (Merck, Darmstadt, Germany) before Sb was quantified by graphite furnace electrothermal atomic absorption spectrometry using an AAnalyst 600/800 spectrometer (Perkin Elmer, Waltham, MA, USA). Blank matrix was established by measuring Sb traces in Sb-unexposed HNO₃-digested cells. Blank absorbance values were subtracted as background. Intracellular Sb content was normalized by number of cells.
Sb-resistant *Leishmania* mutants were maintained at least 2 passages without drug pressure prior to the transport assay to avoid contaminations.

**Statistical analyses**

EC$_{50}$ values were calculated by non-linear regression when applied, data were analyzed by Student’s $t$ test or analysis of variance (ANOVA) followed by correction performed using Bonferroni’s multiple comparison test. A $p$ value $\leq 0.05$ were considered statistically significant. Statistical analyses were carried out using the software GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

**Results**

**In vitro selection of Sb-resistant *L. guyanensis* parasites**

Four independent SbR *L. guyanensis* mutants (LgSb$^{III}$650.1, LgSb$^{III}$650.2, LgSb$^{III}$650.3 and LgSb$^{III}$650.4) were obtained in vitro by stepwise Sb$^{III}$ selection. While *L. guyanensis* wild-type (WT) parasites presented an EC$_{50}$ of 53.72 μM, the four SbR mutants were resistant to at least 1 mM of Sb$^{III}$, representing a resistance index (RI) of more than 18 times (Table 1). The resistance phenotype of every mutant remained stable even after 26 passages in absence of antimonial, at which point parasites still presented Sb$^{III}$ EC$_{50}$ values superior to 1 mM (Table 1).

**Increased gene copy number in *L. guyanensis* SbR mutants is due to aneuploidy and to intrachromosomal amplification**

Whole-genome sequencing was conducted on the four independent *L. guyanensis* SbR lines as well as on the isogenic *L. guyanensis* M4147 WT line by Illumina next-generation sequencing. For all strains, this produced genome assemblies of 31 Mb with a coverage depth of at least 50 fold. Copy number variations (CNVs) associated with resistance were identified by comparing the coverage of uniquely mapped reads between *L. guyanensis* SbR mutants and the WT line as part of small non-overlapping genomic windows (5 kb) along the chromosomes (normalized for the total number of uniquely-mapped reads for each strain) [51]. This enabled the observation of CNVs at the level of entire chromosomes (aneuploidy) and at specific genomic loci (amplification/deletion).

Several cases of supernumerary chromosomes were observed in the SbR mutants (Figs. 1 and S1). Most of these had log$_2$ SbR/WT read ratios close to 0.5 indicating a gain of about 1.5 chromosome copies compared to WT parasites. Parasites from the *Leishmania Viannia* subgenus are distinct from other *Leishmania* species in harboring predominantly trisomic genomes [52] and this should thus represent a gain of one allele compared to WT parasites (going from 3 to 4 chromosome copies). Most supernumerary chromosomes were not shared by the mutants however; chromosome 13 was consistently increased in all SbR mutants, and chromosomes 11 and 25 were increased in three of the four mutants (LgSb$^{III}$650.1, LgSb$^{III}$650.2 and LgSb$^{III}$650.3) (S1 Fig.). Chromosome losses were also observed in the SbR mutants and these were consistent with the loss of one allele (S1 Fig.). Interestingly, CNVs calculated from read depth coverage often led to a cumulative ploidy not matching with a clear-cut number of chromosomes but instead to intermediate log$_2$ SbR/WT values (S1 Fig.). This was observed for both chromosome gains and losses and suggests differences in chromosome-level CNVs between individual cells within the population, a phenomenon known as mosaic aneuploidy [53]. Overall, mutant LgSb$^{III}$650.4 was more divergent and displayed the highest level of chromosome-level CNVs compared to the three other mutants (Fig. 1).
Normalized read depth coverage allowed the identification of amplified and deleted genomic loci in the *L. guyanensis* SbR mutants. These are characterized by punctuated series of genomic windows one beside the other whose normalized read coverage varies compared to the WT baseline [51], as observed for chromosomes 19 and 23 in more than one mutant (S1 Fig.). For chromosome 19, a subtelomeric region of 87.5 kb covering 30 genes (*LbrM*<sub>19.0010</sub> to *LbrM*<sub>19.0300</sub>) appeared to be amplified in mutants LgSbIII650.1, LgSbIII650.2, and LgSbIII650.3 based on normalized read counts (Fig. 2A) and this amplification was confirmed by the hybridization of Southern blots with three distinct probes along the chromosome (Fig. 2A and 2B). Probes derived from genes *LbrM*<sub>19.0270</sub> and *LbrM*<sub>19.0120</sub> (all gene IDs reported in this work are based on the closest *L. braziliensis* genome used for alignments of *L. guyanensis* sequencing reads) located within the amplified region of chromosome 19 yielded 1.6 to 1.9 fold-increase hybridization intensities for mutants LgSbIII650.1, LgSbIII650.2, and LgSbIII650.3 compared to WT cells after normalization with *LbrM*<sub>19.1070</sub> used as an internal control for DNA loading (Fig. 2B). Consistent with the NGS data, mutant LgSbIII650.4 had band intensities equivalent to WT for both

| Strain                  | Sb<sup>III</sup> EC<sub>50</sub> (μM) ± SEM | RI     |
|-------------------------|------------------------------------------|--------|
| *L. guyanensis* M4147 WT| 53.72 ± 0.12                             | >18.6  |
| LgSb<sup>III</sup>650.1 | >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.2 | >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.3 | >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.4 | >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.1 rev| >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.2 rev| >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.3 rev| >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.4 rev| >1000                                    | >18.6  |
| LgSb<sup>III</sup>80.1/2013| 318.4 ± 2.95                            | 6      |
| LgSb<sup>III</sup>80.2/2013| 221.8 ± 2.25                            | 4.12   |
| LgSb<sup>III</sup>160.1/2013| 450.5 ± 2.87                            | 8.4    |
| LgSb<sup>III</sup>160.2/2013| >1000                                    | >18.6  |
| LgSb<sup>III</sup>240.1/2013| 853.4 ± 3.4                             | 15.8   |
| LgSb<sup>III</sup>240.2/2013| 966.3 ± 4.6                             | 18     |
| LgSb<sup>III</sup>325.1/2013| >1000                                    | >18.6  |
| LgSb<sup>III</sup>325.2/2013| >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.1/2013| >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.2/2013| >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.2 + pSP72αZEOαLgAQP1WTGFP| 29.09 ± 0.35                            | 0.54   |
| LgSb<sup>III</sup>650.2 + pSP72αZEOαLgAQP1G133DGFP| >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.2 + pSP72αZEOα| >1000                                    | >18.6  |
| LgSb<sup>III</sup>650.2 + pSP72αZEOαLgAQP1WT| 31.4 ± 0.57                             | 0.6    |
| LgWT + pSP72αZEOαLgAQP1WT| 12.93 ± 0.32                            | 0.24   |
| LgWT + pSP72αZEOαLgAQP1G133D| 51.98 ± 0.33                            | 0.96   |
| LgSb<sup>III</sup>650.4 + pSP72αZEOαLgAQP1WT| 28.63 ± 0.38                            | 0.53   |

SEM (standard error of the mean); RI (resistance index)

*<sup>a</sup> 76% increased Sb<sup>III</sup> sensitivity compared to Lg WT<br>**<sup>b</sup> At least 97% re-sensitization when compared to LgSb<sup>III</sup>650.4

EC<sub>50</sub> values are the average of at least three independent experiments.

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and LbrM.19.0280 (Fig. 2B). Interestingly, for mutants LgSbIII650.1, LgSbIII650.2, and LgSbIII650.3 harboring the subtelomeric amplification on chromosome 19, hybridization of chromosomes separated by PFGE with probes derived from genes LbrM.19.0270 and LbrM.19.0280 revealed a unique band (corresponding to chromosome 19) supporting intrachromosomal duplication of a specific region rather than extrachromosomal elements.

For chromosome 23, the amplified region was much larger than for chromosome 19 and covered 480–495 kb in mutants LgSbIII650.1, LgSbIII650.2, and LgSbIII650.3 (Fig. 3A), starting from one subtelomeric end and encompassing the locus coding for the well-established Sb resistance gene MRPA (LbrM.23.0280) (Fig. 3A and 3B). In mutant LgSbIII650.4, the increased in
read length covers (almost) the entire length of the chromosome (Fig. 3A), suggesting an increased in ploidy. Southern blots hybridization of PstI-digested genomic DNA revealed an up to 1.7 fold increased intensity for a MRPA-derived probe in the LgSbIII650.1–4 mutants after normalization with GAPDH signals used as DNA loading control (Fig. 3B). This is consistent with NGS data that revealed a 1.4–1.7 increased reads counts in the mutants compared to WT parasites (Fig. 3A). Intriguingly, PFGE-derived Southern blots hybridized with MRPA and Lbr.23.1000, two genes comprised in the 480–495 kb region amplified in mutants LgSbIII650.1 to LgSbIII650.3, presented a signal at 785 kb corresponding to chromosome 23 but also an additional signal at around 1.1 Mb (Fig. 3C and 3D). This 1.1 Mb band did not hybridize with
LbrM.23.1660, a probe outside of the 480–495 kb amplified region (Fig. 3E). It is unclear how a region of chromosome 23 found its way to this chromosome. It is unlikely that it presents a linear amplicon as we never observed such large extrachromosomal elements [54,55] and the hybridization intensity (Fig. 3C and 3D) would suggest that this region has translocated into only one of the two homologous recipient chromosome in mutants LgSbIII650.1, LgSbIII650.2 and LgSbIII650.3 (see also S2 Fig.).

Quantitative real time PCR validated that DNA amplification on chromosome 19 and 23 translated into increased mRNA levels (Fig. 4). The four independent mutants presented twice-more mRNA levels for MRPA compared to WT (Fig. 4A) while genes on chromosome 19 were upregulated in mutants LgSbIII650.1, LgSbIII650.2 and LgSbIII650.3, but not in LgSbIII650.4 (Fig. 4B), confirming what was previously observed at the genomic level.

Fig 3. Chromosome 23 amplification in antimony resistant L. guyanensis mutants. (A) Log₂-transformed SbR/WT reads ratios for non-overlapping 5 kb genomic windows on chromosome 23. Blue, LgSbIII650.1; Red, LgSbIII650.2; Green, LgSbIII650.3; and Yellow, LgSbIII650.4. Grey, black and hatched arrows define the location of probes derived from genes MRPA, LbrM.23.1000, LbrM.23.1660 and LbrM.23.1910, respectively, that were used for hybridization of Southern blots in panels B-E. (B) Southern blot hybridization of PstI-digested genomic DNA with a probe derived from LbrM.23.0280 (MRPA). The blot was also probed with GAPDH and used as a loading control. Southern blots of PFGE-separated chromosomes were hybridized with probes derived from genes MRPA (C), LbrM.23.1000 (D) and LbrM.23.1660 (E). Lanes: M, molecular weight marker; 1, LgM4147 WT; 2, LgSbIII650.1; 3, LgSbIII650.2, 4, LgSbIII650.3; 5, LgSbIII650.4.
AQP1-containing locus on chromosome 31 is deleted in three *L. guyanensis* SbR mutants

A fine scale analysis of sequencing coverage revealed that a subtelomeric deletion occurred on chromosome 31 in three of the four SbR *L. guyanensis* mutants (S1 Fig. and Fig. 5A). The deleted region covered around 25 kb in mutants LgSbIII650.1 and LgSbIII650.3 and 27 kb in mutant LgSbIII650.2 (Fig. 5A). In all three mutants the deleted region harbored the gene coding for the aquaglyceroporin AQP1 (*LbrM.31.0020*) known to be associated with antimony uptake in *Leishmania* [27]. Interestingly, sequencing reads could still be detected within 5 kb of the end of chromosome 31 in the mutants that presented AQP1 deletion (Fig. 5A) which could suggest telomere seeding in response to the loss of a terminal part of chromosome 31 (Figs. 5A and S1). The subtelomeric deletion in LgSbIII650.1, LgSbIII650.2 and LgSbIII650.3 was confirmed by hybridization of Southern blots using probes located within and outside the deleted region. As expected, no signal was detected from gene *LbrM.31.0010* to *LbrM.31.0070* in mutants LgSbIII650.1, LgSbIII650.2 and LgSbIII650.3 while LgSbIII650.4 and WT parasites presented a clear AQP1 signal (Fig. 5B). Conversely, hybridization signals were detected for every strain when the blots were probed with gene *LbrM.31.0100* located outside the deleted regions or with the GAPDH gene located on a distinct chromosome (Fig. 5B). Since AQP1 was not deleted in mutant LgSbIII650.4, qRT-PCR assays were carried out in order to infer about any possible regulation of AQP1 expression in this mutant. However, AQP1 mRNA levels were similar in WT and in the LgSbIII650.4 mutant growing in presence of SbIII or in its absence for 26 passages (Fig. 5C). As expected, AQP1 expression was not detected by qRT-PCR in any of the three other mutants (Fig. 5C).

To better understand the kinetics of AQP1 deletion and its implication on growth fitness in the presence and absence of drug pressure, we selected two new series of SbR *L. guyanensis* mutants by five SbIII increments until reaching a final concentration of 650 μM. These series were named LgSbIII.1/2013 and LgSbIII.2/2013 (Table 1). At the first selection step (80 μM) AQP1 remained unaltered in both cell lines (Fig. 6A). When SbIII selection was increased, the AQP1 gene remained intact in the LgSbIII.1/2013 series but was lost in mutant LgSbIII160.2/2013 already at 160 μM (Fig. 6A). The amount of AQP1 mRNA was consistent with the copy number of the gene (Fig. 6B). Growth curves of LgSbIII.1/2013 and LgSbIII.2/2013 mutants revealed an advantage associated with the loss of AQP1 when parasites were cultivated in the presence of SbIII. Indeed, the LgSbIII.2/2013 mutant without AQP1 grew faster under SbIII selection (up to 325 μM) than LgSbIII.1/2013 mutants (Fig. 6D to 6F) presenting intact AQP1 copies (Figs. 6A and S3). This growth advantage of LgSbIII.2/2013 was not observed when parasites were cultures in drug free medium (S4 Fig.).

**Single mutation G133D in AQP1 is involved in antimony resistance in *L. guyanensis***

While the loss of AQP1 allows for a faster acquisition of resistance (Fig. 6), mutant LgSbIII650.4 had intact AQP1 copy number (Fig. 5B) and expression levels (Fig. 5C). Antimony accumulation experiments were thus performed with the *L. guyanensis* mutants. Quantification of intracellular antimony in *L. guyanensis* revealed, as expected, a very low accumulation of metalloid in the *L. guyanensis* SbR mutants in which AQP1 was deleted when compared to WT parasites (Fig. 7). Surprisingly, we also observed low accumulation in LgSbIII650.4 (Fig. 7). We hypothesized that AQP1 in LgSbIII650.4 may be mutated and this was confirmed by sequencing the gene, which revealed a single nucleotide polymorphism (SNP) at AQP1 position 398 in LgSbIII650.4, substituting a guanine for an adenine (S5 Fig.). This missense mutation in AQP1 translated into the replacement of a glycine (Gly) residue by an aspartic acid (Asp) at position 133 (G133D) of the...
protein in LgSbIII650.4 (S6 Fig.). Glycine 133 is putatively located in the third transmembrane domain in LgAQP1 (Fig. 8A) and is conserved among several Leishmania species and also in the Plasmodium falciparum AQP (PfAQP) (Fig. 8B).

To functionally validate the contribution of the AQP1 G133D mutation in antimony resistance in L. guyanensis, GFP-tagged version of AQP1WT and AQP1G133D were episomally maintained in LgSbIII650.2, which is naturally disrupted for AQP1 (Fig. 5B). Hybridization of Western blots with an antibody directed against GFP yielded the expected 50 kDa band for the fusion protein and confirmed the overexpression of the fusion protein in the respective LgSbIII650.2 transfectants (Fig. 9). The overexpression of LgAQP1WT substantially sensitized LgSbIII650.2 to SbIII whose EC50 dropped from more than 1000 μM in the mock-transfected
control, to 29 μM in the presence of the WT AQP1 allele (Table 1). On the other hand, LgSbIII650.2 transfected with an AQP1 version harboring the G133D mutation remained as resistant as the mock-transfected control (Table 1). The presence of GFP did not interfere with the function of AQP1, as the tagged version of WT AQP1 was equally potent as an untagged version of the protein at sensitizing the LgSbIII650.2 mutant to SbIII (Table 1). The WT version of AQP1 but not its mutated version also restored SbIII sensitivity when transfected in LgSbIII650.2 (Table 1). The G133D AQP1 also failed to alter SbIII EC50 when overexpressed in a L. guyanensis WT background (Table 1).

Fig 5. Subtelomeric deletion of chromosome 31 and LgAQP1 expression in antimony resistant L. guyanensis. (A) Zoomed representation of raw read depth for one of the subtelomeric region of chromosome 31. The inset scheme indicates the gene positions on the chromosome. Black, LgM4147 WT; Blue, LgSbIII650.1; Red, LgSbIII650.2; Green, LgSbIII650.3; and Yellow, LgSbIII650.4. (B) Southern blot hybridization validating the subtelomeric deletions of chromosome 31. PstI-digested genomic DNAs were hybridized with probes derived from genes located within (LbrM.31.0010—LbrM.31.0070) or outside (LbrM.31.0100) the deleted region. GAPDH was used as a qualitative DNA loading control for one of the blots and should not be used for determining changes in gene copy numbers. Lanes: 1, LgM4147 WT; 2, LgSbIII650.1; 3, LgSbIII650.2; 4, LgSbIII650.3; 5, LgSbIII650.4. (C) Relative AQP1 mRNA levels in LgSbIII650.1, LgSbIII650.2, LgSbIII650.3 and LgSbIII650.4 and their revertants compared to WT. Revertants were cultured for at least 26 passages in the absence of SbIII. The SbR/WT expression ratios were normalized according to GAPDH (LbrM.30.2950) levels. Values are the mean of at least three independent experiments each performed with three biological replicates.

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Next generation sequencing has been a useful approach for studying drug resistance in *Leishmania* parasites for detecting both point mutations and changes in copy number of genes [30,31,52,56–58]. A frequent mechanism of drug resistance is gene amplification of specific regions that happens at the levels of repeated sequences that abound in the *Leishmania* genomes [55]. Changes in copy number can extend also to whole chromosomes [30,33,59] and it has been argued that tolerance of such chromosomal CNVs may be beneficial under stress conditions as in the presence of drug pressure [60]. Finally the individual parasites within a population may have different specific genes amplified [55] and may have different ploidy of specific chromosomes [53,61]. The NGS technology was useful to detect several ploidy changes in *Leishmania* species and here we have tested it with the
Viannia subgenus. Normalized read depth coverage identified chromosomes in our SbR L. guyanensis mutants whose ploidy was altered compared to WT parasites (Fig. 1). Recurrent changes are often strong candidates for linking a phenotype to a genotype and it is salient to point out that no single chromosome ploidy was identical between the 4 mutants (Fig. 1). The link between aneuploidy and drug resistance might therefore be circumstantial, but antimony resistance is a complex and multifactorial process [29] and, in this context, studying the cellular consequences of aneuploidy might still provide novel insights on drug resistance in Leishmania.

Sequence reads corresponding to a subtelomeric region of chromosome 19 were higher in three mutants out of four (Fig. 2A). This was confirmed by Southern blots, but chromosome sized gels did not support the possibility that this region was amplified as part of an extrachromosomal element and instead consisted in an intrachromosomal duplication (Fig. 2C and 2D). While representing a rare event in Leishmania compared to extrachromosomal amplification, this has already been observed while attempting to inactivate the essential gene GSH1 in Leishmania [62] or in L. major cells resistant to antimony in which an intrachromosomal amplification of a subtelomeric region of chromosome 34 was observed [31]. Species belonging to the Viannia subgenus have previously been reported to display a limited capacity to generate/maintain extrachromosomal DNA [63,64] which is consistent with the intrachromosomal amplifications observed here. Nonetheless, episome transfection is possible in Viannia (see Table 1) [65] and in some studies gene amplification was observed in Viannia parasites [38,66].

Read depth coverage also revealed large regions of chromosome 23 encompassing the MRPA resistance locus that were amplified in the four resistant mutants (Fig. 3A), a feature confirmed by hybridization of Southern blots (Fig. 3B). Karyotype analyses by PFGE revealed

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Fig 7. Intracellular antimony accumulation in L. guyanensis parasites sensitive and resistant to antimony. Antimony quantification was performed using atomic absorption after 1 h incubation of LgWT and LgSbIII650.1–4 parasites with 540 μM of SbIII. Values were obtained from two independent experiments performed in quadruplicate and represent the mean antimony concentration ± SEM. Statistical analysis were carried out using ANOVA followed by Bonferroni’s multiple comparison test. *** p < 0.0001.

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Leishmania guyanensis M4147
A new band of ~1 Mb in three mutants and an apparent change in chromosome ploidy for the fourth mutant (Fig. 3C and 3D). The exact mechanism of formation of the 1 Mb band is unknown but a fragment of 495 kb derived from chromosome 23 must have been rearranged since the MRPA and LbrM probes are 495 kb apart (Fig. 3C and 3D). The hybridization signal to the novel ~1 Mb band in LgSbIII650.1, LgSbIII650.2 and LgSbIII650.3 is less intense than the native chromosome 23 signal at ~800 kb, suggesting that the duplication of the MRPA containing fragment may have happened in only part of the population. Because of the short reads linked to Illumina sequencing, it is not helpful in determining how a portion of chromosome 23 has duplicated into a larger chromosome. One possibility would involve translocation (possibly subtelomeric) from one chromosome to another. While gene amplification (extrachromosomal or intrachromosomal) usually occurs at the level of repeated sequences [55], we have reported rare mechanistic events leading to gene amplification [31] and further studies are required to explain how these MRPA amplifications are produced. These results are consistent with the data shown above for the subtelomeric region of chromosome 19 where in Viannia, in comparison to Leishmania, increase in copy number is mediated by mechanisms that do not involve extrachromosomal amplification.

A terminal deletion of ~20 kb of seven genes on chromosome 31 including the gene coding for AQP1 was observed in three mutants (Fig. 5A and B). AQP1 is considered the major route of entry of trivalent antimony in Leishmania [27] and its overexpression leads to SbIII hypersensitivity [26,27]. Downregulation of AQP1 has been observed in both laboratory-raised and clinical Leishmania parasites resistant to antimony [26,31,67,68] and constitutes a potentially
Fig 9. Analysis of LgAQP1-GFP expression by Western blot. Anti-GFP monoclonal antibody was used to confirm the expression of the LgAQP1WT-GFP and LgAQP1G133D-GFP fusion proteins from protein extracts prepared from LgSbIII650.2 parasites transfected with pSP72αZEOαLgAQP1WT (lane 1), pSP72αZEOαLgAQP1G133D (lane 2) or pSP72αZEOα (lane 3). Anti-α-tubulin antiserum was used as a loading control.

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useful biomarker for antimony resistance. Deletion of AQPI is also a major contributor to SbR in L. guyanensis because episomal overexpression of a WT AQPI allele was sufficient to restore Sb sensitivity (Table 1) and accumulation (Fig. 7) in every mutant tested. The deletion of the gene also suggests that AQPI is not essential in L. guyanensis. Chromosome 31 is polyploid in all Leishmania spp. tested [30,52,60] and the 20 kb region was deleted from all chromosome copies (Fig. 5B). Terminal deletions from 67 to 205 kb covering the AQPI locus on chromosome 31 were also recently observed in SbR L. major for which the break points occurred at the level of inverted repeated sequences [31]. In these cases however, there was still one intact copy of AQPI. In contrast, L. guyanensis SbR mutants presented deleted regions of only 20.7 to 22.7 kb on chromosome 31 without any sign of inverted repeated sequences. The terminal deletion could thus be driven by micro-homologies with telomere-associated repeated sequences [31,69] or through a double strand break followed by a terminal healing process driven by telomere seeding [70], although any of these mechanisms need to be ascertain.

In one mutant, the AQPI gene was not deleted but transport experiments indicated that there was no accumulation of SbIII (Fig. 7). This prompted us to sequence AQPI and to demonstrate for the first time that a point mutation in AQPI (G133D) can also be a novel resistance mechanism. Mutational analysis on LmAQPI had already revealed that residues located at C-loop Ala163 and Glu152 (equivalent to LgAQPI residues Val167 and Glu156, respectively—Fig. 8A) are involved in metalloid uptake and reduced permeability to antimony [28,71]. LmAQPI is also post-transcriptionally regulated by a mitogen activated protein kinase 2-mediated phosphorylation at Thr197 (LgAQPI Thr201—Fig. 8A) modulating SbIII uptake and sensitivity [72]. The absence of a crystal structure for Leishmania AQPI precludes hypothesizing about the precise role of G133D in resistance. The lack of antibodies and the low level of GFP fluorescence have not allowed to test whether the G133D mutation could also impact the subcellular localization of AQPI. Interestingly, the homolog of LgAQPI in the related protozoan Trypanosoma brucei (TbAQPI) was also linked to resistance to the arsenical-based compound melarsoprol (arsenic is a metalloid chemically related to antimony) [73,74]. Melarsoprol-resistant T. brucei were shown to have lost TbAQPI or to harbor a nonfunctional chimera derived from recombination events between TbAQPI and TbAQP3 [75] which show similarity with the mutants studied here (deletion or point mutations). Given the important role of aquaglyceroporins in volume and osmo-taxis regulation [25], how null mutants compensate these functions is still an open question and further studies will be required for understanding their physiological adaptations.

Every L. guyanensis SbR mutants had a defect in antimony accumulation. Lower accumulation can be achieved either through decreased uptake or increased efflux. An additional transport mechanisms leading to resistance would be drug sequestration mediated by the intracellular ABC protein MRPA [36]. The lack of a functional AQPI will lead to reduced uptake. Possibly minimal amounts of antimony can enter by other routes and overexpression of MRPA can lead to drug sequestration. Alternatively, the contribution of MRPA to resistance may be more important in early selection steps when all AQPI copies (chromosome 31 harbouring AQPI is polyploid in every Leishmania species) have not yet been inactivated/deleted. It is salient to point out that alterations in expression of MRPA and AQPI has been described in antimony-resistant natural isolates of L. donovani [29,67,68] and L. tropica [76], suggesting a concomitant antimony sequestering and decreased uptake [67,76]. These results are also consistent with antimony resistant L. amazonensis mutants selected in vitro [38]. The loss of AQPI appears to be dominant in our current mutants since providing the mutants with a functional version of AQPI enables complete re-sensitization to SbIII.

The present study highlighted that similar markers are involved in resistance in Leishmania and Viannia subgenus but that gene amplification differs with mostly extrachromosomal ampli-cons in Leishmania and intrachromosomal ones in Viannia. A new resistance mechanism
corresponding to a point mutation in AQP1 was also discovered and this will allow further testing of the role of AQP1 in resistance.

Supporting Information

S1 Fig. Normalized SbR/WT reads ratios per non-overlapping 5kb genomic window for the 35 chromosomes of *L. guyanensis*. Chromosomes were divided into non-overlapping 5kb genomic windows and for each window the SbR/WT reads ratios (normalized to the total number of reads per samples) were plotted as log2-transformed values according to chromosome positions. Blue, LgSbIII650.1; Red, LgSbIII650.2; Green, LgSbIII650.3; and Yellow, LgSbIII650.4. (ZIP)

S2 Fig. Scheme representation of the MRPA locus on chromosome 23. Grey and black arrows indicate pairs of inverted repeats that could anneal to prime the inverted duplication of the large 495 kb region. Black arrows annealing would result in a 1,032 kb linear element while grey arrows would lead to a 1,123 kb linear amplicon. (TIF)

S3 Fig. Multiple nucleotide alignment of AQP1 from the LgSbIII.1/2013 series of mutants. AQP1 sequences were compared to *L. guyanensis* AQP1 GenBank accession number GU368155.1 (LgAQP1_GB). Alignment was performed using the MultiAlin interface (multialin.toulouse.inra.fr). (TIF)

S4 Fig. Growth curves of LgSbIII/2013 mutants and Lg M4147 WT in absence of SbIII. The growth of LgSbIII.1/2013 and LgSbIII.2/2013 mutants resistant to 80 μM (A), 160 μM (B), 240 μM (C), 325 μM (D) and 650 μM (E) SbIII was monitored without drug pressure for 8 days. The growth of the parental LgM4147 WT line was also monitored. An asterisk (*) indicates comparisons between LgSbIII.1/2013 and LgSbIII.2/2013 mutants. Values represent the average of two independent growth measurements performed in duplicate. Statistical analysis was carried out using Student’s t-test. * $p \leq 0.05$ and ** $p \leq 0.01$. (TIF)

S5 Fig. Multiple nucleotide alignment of AQP1 from *L. guyanensis* M4147 WT and LgSbIII650.4. Guanine 398 is replaced by an adenine in LgSbIII650.4. The *L. guyanensis* AQP1 sequence GU368155.1 (strain MHOM/BR/1997/NMT-MAO 328P clone B) was used as an additional reference [81]. Alignment was performed with ClustalW2 and plotted using TEXshade [79,80]. *L. guyanensis* AQP1 sequences are available in GenBank, accession numbers KJ623262 and KJ623263. (TIF)

S6 Fig. Multiple alignment of AQP1 protein sequences from *L. guyanensis* M4147 WT and LgSbIII650.4. The substitution of a glycine by an aspartic acid at position 133 (G133D) is associated with antimony resistance in mutant LgSbIII650.4. The *L. guyanensis* AQP1 sequence GU368155.1 was used as an additional reference [81]. Alignment was performed using ClustalW2 and plotted using TEXshade [79,80]. (TIF)

S1 Table. List of PCR primers used in this study. (TIF)

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Author Contributions
Conceived and designed the experiments: RMN PL FF MO. Performed the experiments: RMN MCNL PR. Analyzed the data: RMN PL FF MO. Contributed reagents/materials/analysis tools: PL FF. Wrote the paper: RMN PL MO.

References
1. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, et al. (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7: e35671. doi: 10.1371/journal.pone.0035671 PMID: 22693548
2. McCall LI, Zhang WW, Matlashewski G (2013) Determinants for the development of visceral leishmaniasis disease. PLoS Pathog 9: e1003053. doi: 10.1371/journal.ppat.1003053 PMID: 23300451
3. Santrich C, Segura I, Arias AL, Saravia NG (1990) Mucosal disease caused by Leishmania braziliensis guyanensis. Am J Trop Med Hyg 42: 51–55. PMID: 2301705
4. Guerra JA, Prestes SR, Silveira H, Coelho LI, Gama P, et al. (2011) Mucosal Leishmaniasis caused by Leishmania (Viannia) braziliensis and Leishmania (Viannia) guyanensis in the Brazilian Amazon. PLoS Negl Trop Dis 5: e980. doi: 10.1371/journal.pntd.0000980 PMID: 21408116
5. Figueroa RA, Lozano LE, Romero IC, Cardona MT, Prager M, et al. (2009) Detection of Leishmania in unaffected mucosal tissues of patients with cutaneous leishmaniasis caused by Leishmania (Viannia) species. J Infect Dis 200: 638–646. doi: 10.1086/600109 PMID: 19569974
6. Reveiz L, Maia-Elkhoury AN, Nicholls RS, Romero GA, Yadon ZE (2013) Interventions for American cutaneous and mucocutaneous leishmaniasis: a systematic review update. PLoS One 8: e61843. doi: 10.1371/journal.pone.0061843 PMID: 23637917
7. Mutiso JM, Macharia JC, Kiio MN, Ichagichu JM, Rikoi H, et al. (2013) Development of Leishmania vaccines: predicting the future from past and present experience. J Biomed Res 27: 85–102. doi: 10.7555/JBR.27.20120064 PMID: 23554800
8. Sosa N, Capitan Z, Nieto J, Nieto M, Calzada J, et al. (2013) Randomized, double-blinded, phase 2 trial of WR 279,396 (paromomycin and gentamicin) for cutaneous leishmaniasis in Panama. Am J Trop Med Hyg 89: 557–563. doi: 10.4269/ajtmh.12-0736 PMID: 23857024
9. Apa H, Devrim I, Bayram N, Deveci R, Demir Ozek G, et al. (2013) Liposomal amphotericin B versus pentavalent antimony salts for visceral Leishmania in children. Turk J Pediatr 55: 378–383. PMID: 24292030
10. Berman JJ (2008) Treatment of leishmaniasis with miltefosine: 2008 status. Expert Opin Drug Metab Toxicol 4: 1209–1216. doi: 10.1517/17425255.4.9.1209 PMID: 18721114
11. Haldar AK, Sen P, Roy S (2011) Use of antimony in the treatment of leishmaniasis: current status and future directions. Mol Biol Int 2011: 571242. doi: 10.4061/2011/571242 PMID: 22091408
12. Sundar S, Chakravarty J (2013) Leishmaniasis: an update of current pharmacotherapy. Expert Opin Pharmacother 14: 53–63. doi: 10.1517/14656566.2013.755515 PMID: 23256601
13. Peters W (1981) The treatment of kala-azar—new approaches to an old problem. Indian J Med Res 73 Suppl: 1–18. PMID: 7021410
14. Oliveira-Neto MP, Schubach A, Mattos M, Goncalves-Costa SC, Pirmez C (1997) A low-dose antimony treatment in 159 patients with American cutaneous leishmaniasis: extensive follow-up studies (up to 10 years). Am J Trop Med Hyg 57: 651–655. PMID: 9430521
15. Romero GA, Guerra MV, Paes MG, Macedo VO (2001) Comparison of cutaneous leishmaniasis due to Leishmania (Viannia) braziliensis and L. (V.) guyanensis in Brazil: therapeutic response to meglumine antimoniate. Am J Trop Med Hyg 65: 456–465. PMID: 11716098
16. Arevalo J, Ramírez L, Adau V, Zimic M, Tuljiano G, et al. (2007) Influence of Leishmania (Viannia) species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. J Infect Dis 195: 1846–1851. PMID: 17492601
17. Palacios R, Osorio LE, Grajalera LF, Ochoa MT (2001) Treatment failure in children in a randomized clinical trial with 10 and 20 days of meglumine antimonate for cutaneous leishmaniasis due to Leishmania viannia species. Am J Trop Med Hyg 64: 187–193. PMID: 11442216
18. Rojas R, Valderrama L, Valderrama M, Varona MX, Ouellette M, et al. (2006) Resistance to antimony and treatment failure in human Leishmania (Viannia) infection. J Infect Dis 193: 1375–1383. PMID: 16619185
19. Yardley V, Ortuno N, Llanos-Cuentas A, Chappuis F, Doncker SD, et al. (2006) American tegumentary leishmaniasis: is antimonial treatment outcome related to parasite drug susceptibility? J Infect Dis 194: 1168–1175. PMID: 16991093
20. Shaked-Mishan P, Ulrich N, Ephros M, Zilberstein D (2001) Novel Intracellular SbV reducing activity correlates with antimony susceptibility in Leishmania donovani. J Biol Chem 276: 3971–3976. PMID: 11110784

21. Ferreira CS, Martins PS, Demicheli C, Brochu C, Ouellette M, et al. (2003) Thiol-induced reduction of antimony(V) into antimony(III): a comparative study with trypanothione, cysteinyl-glycine, cysteine and glutathione. Biometals: an international journal on the role of metal ions in biology, biochemistry, and medicine 16: 441–446.

22. Zhou Y, Messier N, Ouellette M, Rosen BP, Mukhopadhyay R (2004) Leishmania major LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug pentostam. J Biol Chem 279: 37445–37451. PMID:15220340

23. Bhattacharjee H, Rosen BP, Mukhopadhyay R (2009) Aquaglyceroporins and metalloid transport: implications in human diseases. Handb Exp Pharmacol doi:10.1007/978-3-540-79885-9_16: 309–325. PMID: 19096785

24. Ramirez-Solis A, Mukopadhyay R, Rosen BP, Stemmler TL (2004) Experimental and theoretical characterization of arsenite in water: insights into the coordination environment of As-O. Inorg Chem 43: 2954–2959. PMID:15106984

25. Figarella K, Uzcategui NL, Zhou Y, LeFurgey A, Ouellette M, et al. (2007) Biochemical characterization of Leishmania major aquaglyceroporin LmAQP1: possible role in volume regulation and osmotaxis. Mol Microbiol 65: 1006–1017. PMID:17640270

26. Gourbal B, Sonuc N, Bhattacharjee H, Legare D, Sundar S, et al. (2004) Drug uptake and modulation of drug resistance in Leishmania by an aquaglyceroporin. J Biol Chem 279: 31010–31017. PMID:15138256

27. Marquis N, Gourbal B, Rosen BP, Mukhopadhyay R, Ouellette M (2005) Modulation in aquaglyceroporin AQP1 gene transcript levels in drug-resistant Leishmania. Mol Microbiol 57: 1690–1699. PMID:16135234

28. Downing T, Imamura H, Decuypere S, Clark TG, Coombs GH, et al. (2011) Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. Genome Res 21: 2143–2156. doi: 10.1101/gr.123430.111 PMID: 22038251

29. Mukherjee A, Boisvert S, Monte-Neto RL, Coelho AC, Raymond F, et al. (2013) Telomeric gene deletion and intrachromosomal amplification in antimony-resistant Leishmania. Mol Microbiol 88: 189–202. doi:10.1111/mmi.12178 PMID: 23421749

30. Leprohon P, Legare D, Raymond F, Madore E, Hardiman G, et al. (2009) Gene expression modulation is associated with gene amplification, supernumerary chromosomes and chromosome loss in antimony-resistant Leishmania infantum. Nucleic Acids Res 37: 1387–1399. doi: 10.1093/nar/gkn1069 PMID: 19192366

31. Haimeur A, Brochu C, Genest P, Papadopoulou B, Ouellette M (2000) Amplification of the ABC transporter gene PGPA and increased trypanothione levels in potassium antimony tartrate (SbIII) resistant Leishmania tarentolae. Mol Biochem Parasitol 108: 131–135. PMID:10802326

32. Legaré D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, et al. (2001) The Leishmania ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase. J Biol Chem 276: 26301–26307. PMID:11306588

33. Walker J, Gongora R, Vasquez JJ, Drummelsmith J, Burchmore R, et al. (2012) Discovery of factors linked to antimony resistance in Leishmania panamensis through differential proteome analysis. Mol Biochem Parasitol 183: 166–176. doi:10.1016/j.molbiopara.2012.03.002 PMID: 22449941

34. Moreira DS, Monte-Neto RL, Andrade JM, Santi AMM, Reis PG, et al. (2013) Molecular characterization of the MRPA transporter and antimony uptake in four New World Leishmania spp. susceptible and...
resistant to antimony. International Journal for Parasitology: Drugs and Drug Resistance 3: 143–153. doi: 10.1016/j.ijpddr.2013.08.001 PMID: 24533304
39. Ouellette M, Fase-Fowler F, Borst P (1990) The amplified H circle of methotrexate-resistant leishmania tarentolae contains a novel P-glycoprotein gene. EMBO J 9: 1027–1033. PMID: 1969794
40. Aslett M, Aurrecoechea C, Berriman M, Brestelli J, Brunk BP, et al. (2010) TriTrypDB: a functional genomic resource for the Trypanosomatidae. Nucleic Acids Res 38: D457–462. doi: 10.1093/nar/gkp851 PMID: 19843604
41. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25: 1754–1760. doi:10.1093/bioinformatics/btp324 PMID: 19451168
42. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, et al. (2009) The Sequence Alignment/Map format and SAMtools. Bioinformatics 25: 2078–2079. doi:10.1093/bioinformatics/btp352 PMID: 19505943
43. Bastien P, Blaineau C, Pages M (1992) Molecular karyotype analysis in Leishmania. Subcell Biochem 18: 131–187. PMID: 1485351
44. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a laboratory manual; ed. n, editor. Cold Spring Harbor: New York: Cold Spring Harbor Laboratory Press. 545 p. PMID: 25144100
45. Papadopoulou B, Roy G, Ouellette M (1992) A novel antifolate resistance gene on the amplified H circle of Leishmania. EMBO J 11: 3601–3608. PMID: 1396560
46. Richard D, Leprohon P, Drummelsmith J, Ouellette M (2004) Growth phase regulation of the main folate transporter of Leishmania infantum and its role in methotrexate resistance. J Biol Chem 279: 54494–54501. PMID: 15466466
47. Mukherjee A, Roy G, Guimond C, Ouellette M (2009) The gamma-glutamylcysteine synthetase gene of Leishmania is essential and involved in response to oxidants. Mol Microbiol 74: 914–927. doi: 10.1111/j.1365-2958.2009.06907.x PMID: 19818018
48. Gamarro F, Chiquero MJ, Amador MV, Legare D, Ouellette M, et al. (1994) P-glycoprotein overexpression in methotrexate-resistant Leishmania tropica. Biochem Pharmacol 47: 1939–1947. PMID: 7912069
49. Roberts WL, Rainey PM (1993) Antimony quantification in Leishmania by electrothermal atomic absorption spectroscopy. Anal Biochem 211: 1–6. PMID: 8323020
50. Wang J, Brochu C, Wang X, Wang N, Ouellette M (2003) Determination of trace antimony in cells by inductively coupled plasma mass spectrometry for drug resistance study of protozoan parasite. Chin J Anal Lab 22: 107–112.
51. Chiang DY, Getz G, Jaffe DB, O’Kelly MJ, Zhao X, et al. (2009) High-resolution mapping of copy-number alterations with massively parallel sequencing. Nat Methods 6: 99–103. doi: 10.1038/nmeth.1276 PMID: 19043412
52. Rogers MB, Hilley JD, Dickens NJ, Wilkes J, Bates PA, et al. (2011) Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania. Genome Res 21: 2129–2142. doi:10.1101/gr.129445.111 PMID: 22038252
53. Sterkers Y, Lachaud L, Crobu L, Bastien P, Pages M (2011) FISH analysis reveals aneuploidy and continual generation of chromosomal mosaicism in Leishmania major. Cell Microbiol 13: 274–283. doi: 10.1111/j.1462-5822.2010.01534.x PMID: 20964798
54. Grondin K, Kundig C, Roy G, Ouellette M (1998) Linear amplicons as precursors of amplified circles in methotrexate-resistant Leishmania tarentolae. Nucleic Acids Res 26: 3372–3378. PMID: 9649621
55. Ubeda JM, Raymond F, Mukherjee A, Plourde M, Gingras H, et al. (2014) Genome-wide stochastic adaptive DNA amplification at direct and inverted DNA repeats in the parasite Leishmania. PLoS biology 12: e1001868. doi: 10.1371/journal.pbio.1001868 PMID: 24844805
56. Coelho AC, Boisvert S, Mukherjee A, Leprohon P, Corbeil J, et al. (2012) Multiple mutations in heterogeneous miltefosine-resistant Leishmania major population as determined by whole genome sequencing. PLoS Negl Trop Dis 6: e1512. doi: 10.1371/journal.pntd.0001512 PMID: 22348164
57. Ritt JF, Raymond F, Leprohon P, Legare D, Corbeil J, et al. (2013) Gene Amplification and Point Mutations in Pyrimidine Metabolic Genes in 5-Fluorouracil Resistant Leishmania infantum. PLoS Negl Trop Dis 7: e2564. doi: 10.1371/journal.pntd.0002564 PMID: 24278495
58. Brotherton MC, Bourassa S, Leprohon P, Legare D, Poirier GG, et al. (2013) Proteomic and genomic analyses of antimony resistant Leishmania infantum mutant. PLoS One 8: e81899. doi: 10.1371/journal.pone.0081899 PMID: 24312377
59. Ubeda JM, Legare D, Raymond F, Ouameur AA, Boisvert S, et al. (2008) Modulation of gene expression in drug resistant Leishmania is associated with gene amplification, gene deletion and chromosome aneuploidy. Genome Biol 9: R115. doi: 10.1186/gb-2008-9-7-r115 PMID: 18638379
60. Mannaert A, Downing T, Imamura H, Dujardin JC (2012) Adaptive mechanisms in pathogens: universal aneuploidy in Leishmania. Trends Parasitol 28: 370–376. doi: 10.1016/j.pt.2012.06.003 PMID: 22789456

61. Lachaud L, Bourgeois N, Kuk N, Morelle C, Crobu L, et al. (2014) Constitutive mosaic aneuploidy is a unique genetic feature widespread in the Leishmania genus. Microbes and infection / Institut Pasteur 16: 61–66. doi: 10.1016/j.micinf.2013.09.005 PMID: 24120456

62. Mukherjee A, Langston LD, Ouellette M (2011) Intrachromosomal tandem duplication and repeat expansion during attempts to inactivate the subtelomeric essential gene GSH1 in Leishmania. Nucleic Acids Res 39: 7499–7511. doi: 10.1093/nar/gkr494 PMID: 21693561

63. Dias FC, Ruiz JC, Lopes WC, Squina FM, Renzi A, et al. (2007) Organization of H locus conserved repeats in Leishmania (Viannia) braziliensis correlates with lack of gene amplification and drug resistance. Parasitol Res 101: 667–676. PMID: 17393181

64. Lye LF, Owens K, Shi H, Murta SM, Vieira AC, et al. (2010) Retention and loss of RNA interference pathways in trypanosomatid protozoans. PLoS Pathog 6: e1001161. doi:10.1371/journal.ppat.1001161 PMID: 21060810

65. Nuhs A, Schafer C, Zander D, Trube L, Jejera Nevaio P, et al. (2014) A novel marker, ARM58, confers antimony resistance to Leishmania spp. Int J Parasitol Drugs Drug Resist 4: 37–47. doi: 10.1016/j.ipddr.2013.11.004 PMID: 24596667

66. Anacleto C, Abdo MC, Ferreira AV, Murta SM, Romanha AJ, et al. (2003) Structural and functional analysis of an amplification containing a PGPA gene in a glucantime-resistant Leishmania (Viannia) guyanensis cell line. Parasitol Res 90: 110–118. PMID: 11526844

67. Rai S, Bhaskar, Goel SK, Nath Dwivedi U, Sundar S, et al. (2013) Role of efflux pumps and intracellular thios in natural antimony resistant isolates of Leishmania donovani. PLoS Pathog 9: e74862. doi: 10.1371/journal.ppat.1000327 PMID: 19180184

68. Tamar S, Papadopoulou B (2001) A telomere-mediated chromosome fragmentation approach to assess mitotic stability and ploidy alterations of Leishmania chromosomes. J Biol Chem 276: 11662–11673. PMID: 11526844

69. Uzcategui NL, Zhou Y, Figarella K, Ye J, Mukhopadhyay R, et al. (2008) Alteration in glycerol and maltoloid permeability by a single mutation in the extracellular C-loop of Leishmania major aquaglyceroporin LmAQP1. Mol Microbiol 70: 1477–1486. doi: 10.1111/j.1365-2958.2008.06494.x PMID: 19019150

70. Alsford S, Eckert S, Baker N, Glover L, Sanchez-Flores A, et al. (2012) High-throughput decoding of antitrypanosomal drug efficacy and resistance. Nature 482: 232–236. doi: 10.1038/nature10771 PMID: 22278056

71. Baker N, Glover L, Munday JC, Aguinaga Andres D, Barrett MP, et al. (2012) Aquaglyceroporin 2 controls susceptibility to melarsoprol and pentamidine in African trypanosomae. Proc Natl Acad Sci U S A 109: 10996–11001. doi: 10.1073/pnas.1202885109 PMID: 22718116

72. Graf FE, Ludin P, Wenzler T, Kaiser M, Brun R, et al. (2013) Aquaporin 2 mutations in Trypanosoma brucei gambiense field isolates correlate with decreased susceptibility to pentamidine and melarsoprol. PLoS Negl Trop Dis 7: e2475. doi: 10.1371/journal.pntd.0002475 PMID: 24130910

73. Kazemi-Rad E, Mohebali M, Khadem-Erfan MB, Saffari M, Raofian R, et al. (2013) Identification of antimony resistance markers in Leishmania tropica field isolates through a cDNA-AFLP approach. Experimental parasitology 135: 344–349. doi: 10.1016/j.exppara.2013.07.018 PMID: 23928349

74. Beitz E, Pavlovic-Djuramovic S, Yasui M, Agre P, Schultz JE (2004) Molecular dissection of water and glycerol permeability of the aquaglyceroporin from Plasmodium falciparum. Mol Microbiol 52: 112–121. doi: 10.1111/j.1365-2958.2004.03849.x PMID: 15130234

75. Newby ZE, O'Connell J 3rd,Robles-Coinmana Y, Khademi S, Miercke LJ, et al. (2008) Crystal structure of the aquaglyceroporin PIAQP from the malarial parasite Plasmodium falciparum. Nat Struct Mol Biol 15: 619–625. doi: 10.1038/nsmb.1431 PMID: 18500352
79. Beitz E (2000) T(E)Xtopo: shaded membrane protein topology plots in LATEX2epsilon. Bioinformatics 16: 1050–1051. PMID: 11159320

80. Beitz E (2000) TEXshade: shading and labeling of multiple sequence alignments using LATEX2 epsilon. Bioinformatics 16: 135–139. PMID: 10842735

81. Torres DC, Ribeiro-Alves M, Romero GA, Davila AM, Cupolillo E (2013) Assessment of drug resistance related genes as candidate markers for treatment outcome prediction of cutaneous leishmaniasis in Brazil. Acta Trop 126: 132–141. doi: 10.1016/j.actatropica.2013.02.002 PMID: 23416123