MRPA-independent mechanisms of antimony resistance in *Leishmania infantum*

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\(\text{ABSTRACT}\)

Control of both human and canine leishmaniasis is based on a very short list of chemotherapeutic agents, headed by antimonial derivatives (Sb). The utility of these molecules is severely threatened by high rates of drug resistance. The ABC transporter MRPA is one of the few key Sb resistance proteins described to date, whose role in detoxification has been thoroughly studied in *Leishmania* parasites. Nonetheless, its rapid amplification during drug selection complicate the discovery of other mechanisms potentially involved in Sb resistance. In this study, stepwise drug-resistance selection and next-generation sequencing were combined in the search for novel Sb resistance mechanisms deployed by parasites when MRPA is abolished by targeted gene disruption. The gene *mrpA* is not essential in *L. infantum*, and its disruption leads to an Sb hypersensitive phenotype in both promastigotes and amastigotes. Five independent *mrpA*\(^{-}\) mutants were selected for antimony resistance. These mutants displayed major changes in their ploidy, as well as extrachromosomal linear amplifications of the subtelomeric region of chromosome 23, which includes the genes coding for ABCB1 and ABCB2. Overexpression of ABCB2, but not of ABCB1, resulted in increased Sb tolerance in the *mrpA*\(^{-}\) mutant. SNP analyses revealed three different heterozygous mutations in the gene coding for a serine acetyltransferase (SAT) involved in de novo cysteine synthesis in *Leishmania*. Overexpression of sat\(^{G321R}\), sat\(^{G325R}\) and sat\(^{G325R}\) variants led to a 2–3.2-fold increase in Sb resistance in *mrpA*\(^{-}\) parasites. Only sat\(^{G321R}\) and sat\(^{G325R}\) induced increased Sb resistance in wild-type parasites. These results reinforce and expand knowledge on the complex nature of Sb resistance in *Leishmania* parasites.

1. Introduction

The protozoan parasite *Leishmania infantum* causes visceral leishmaniasis, a grave systemic disease that affects the spleen, liver, and bone marrow, leading to weight loss, anemia, and even death if left untreated (WHO, 2013; Sundar and Rai, 2002). *Leishmania* are intracellular parasites with a promastigote and an amastigote form, found in the sandfly vector and vertebrate host, respectively (Gazanion et al., 2016). Dogs serve as the main reservoir for infection (Abranches et al., 1991). With over 500,000 new cases of visceral leishmaniasis every year, this widespread disease is responsible for a significant health, psychosocial, and economic burden around the world (WHO, 2013; Sundar and Rai, 2002).

Leishmaniasis has long been treated with antimonial drugs; first trivalent antimonials (Sb\(^{III}\)), and later with the less toxic pentavalent antimonials (Sb\(^{V}\)) (Ouellette and Papadopoulou, 1993). The limited arsenal of available anti-leishmanial therapies coupled with the lack of an effective human vaccine make antimonials a first-line treatment still today – despite their harmful side effects (Ouellette and Papadopoulou, 1993; Papadopoulou et al., 1994; Ouellette et al., 2004). Resistance has become rampant in the field, rendering these drugs virtually ineffective against visceral leishmaniasis in certain areas of the world (Leprohon et al., 2015; Sundar et al., 2000; Rijal et al., 2003).

The mechanism of action of antimonial drugs against *Leishmania* remains ambiguous, further complicating the fight against drug resistance (Ouellette and Papadopoulou, 1993; Légard et al., 2001;
Evidence suggests that pentavalent antimony (SbV) is actually a prodrg, reduced to the active trivalent form (SbIII) in the macrophage and/or parasite (Ouellette et al., 2004). Resistance mechanisms likely rely on decreased accumulation of antimony within the parasite (either through increased efflux or reduced uptake) (Ouellette and Papadopoulou, 1993; Ouellette et al., 2004). Early experiments demonstrated the presence of an efflux pump in the membrane of L. tarentolae that allowed extrusion of metals conjugated to thiols, and similar systems were later observed in L. infantum and L. panamensis (Ouellette et al., 2004; Dey et al., 1996). Although metal resistance in Leishmania spp. is multifactorial, the ATP-binding cassette protein MRPA has been shown to be implicated through recognition of thiol-conjugated metals and subsequent sequestration near the flagellar pocket for later exocytosis and/or extrusion outside the cell (Légaré et al., 2001; El Faridi et al., 2005). Gene amplification is a frequent phenomenon in metal-resistant Leishmania in response to antimional drug pressure; amplifications have been reported both intrachromosomally and as part of ampiclons (Mukherjee et al., 2013; Grondin et al., 1997; Leprohon et al., 2009a; Haimeur et al., 2000). Amplifications of mrpA, often identified within circular ampiclons in antimony-resistant strains, have been extensively characterized (Leprohon et al., 2009a; Mukherjee et al., 2007). However, while an mrpA C−/− has been achieved in L. tarentolae (Papadopoulou et al., 1996), there is no available literature describing the properties of a null mrpA mutant in human pathogenic Leishmania.

The goal of this study was to complete an in-depth exploration of the effects caused by the disruption of the mrpA gene in L. infantum, including its effect on parasite survival, impact on infectivity, and response to antimony sensitivity and selection. This information is critical to being better equipped in the fight against drug-resistant Leishmania by furthering knowledge of one of the many mechanisms these parasites use to survive in the presence of drugs.

2. Material and methods

2.1. Leishmania cultures

Wildtype parasites (WT), as well as the different mutants (e.g. nulls and overexpressors), were cultured as promastigotes at 25 °C in SDM-79 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 5 μg/mL hemin. THP-1 cells (ATCC TIB-202) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU of penicillin/mL, and 100 μg of streptomycin/mL. Prior to infection, log-phase THP-1 cells were differentiated by incubation for 2 days in RPMI 1640 medium containing 20 ng/mL of phorbol myristate acetate (PMA) (Sigma).

2.2. Genomic modifications

2.2.1. mrpA null mutant

The L. infantum mrpA (LinJ.23.0290) null mutant (mrpA C−/−) was obtained by targeted gene replacement. mrpA flanking regions were amplified from L. infantum 263 WT genomic DNA and fused to the puromycin acetyltransferase (puro) gene using a PCR fusion-based method. Briefly, the 5′UTR of mrpA was amplified using primers MRPA_A and MRPA_B, which includes a tail of 25 nucleotides complementary to the puro gene (see primer sequences in Table S1). The puroinformation is critical to being better equipped ORF was amplified with primers MRPA_C and MRPA_D, which includes a tail of 25 nucleotides complementary to the 3′UTR of mrpA. The 3′UTR of mrpA was amplified using primers MRPA_E and MRPA_F. The 5′UTR of mrpA was then ligated to the puromycin resistance marker gene by PCR using primers A and D, information is critical to being better equipped giving the fragment 5′UTR-puro with a tail corresponding to the first 25 bases of the 3′UTR of mrpA. The marker gene was fused with the 3′UTR of mrpA, forming puro-3′UTR using primers C and F. The fragment 5′UTR-puro was then fused to the puro-3′UTR by PCR using primers A and F yielding the recombinant cassette 5′UTR-puro-3′UTR. 10 μg of the 5′UTR-puro-3′UTR linear fragment were transfected by electroporation into L. infantum WT to replace both mrpA alleles. Recombinants were selected in the presence of 200 μg/mL of puromycin dihydrochloride (Wisent). After 4–5 passages, cells resistant to the drug selection were cloned in SDM-Agar plates (1%), also in the presence of 200 μg/mL puromycin. The puro/puro substitution of the mrpA gene was confirmed by PCR analysis of the recombinants and Southern blot. Two different PCRs were used: internal PCR was performed using primer set aa’ that amplifies a 200-bp mrpA-specific region; external PCR was performed using a forward primer located in the mrpA 5′ flanking region with a reverse primer in the mrpA 3′ flanking region (primer set bb’).

2.2.2. mrpA addback and overexpression mutants

Genes mrpA (LinJ.23.0290), ABCBC1 (LinJ.23.0239), ABCBC2 (LinJ.23.0240) and sat (LinJ.34.2710) were amplified from L. infantum genomic DNA using compatible primer pairs (see Table S1) and PCR fragments were ligated into pGEM T-easy (Promega) to confirm the quality of the insert by standard sequencing. PCR fragments were then cloned in the Leishmania expression vector pSP72 chyga (Papadopoulou et al., 1992), which contains the gene hygromycin phosphotransferase (hgy), a selectable marker in Leishmania. A total of 20 μg of plasmid DNA for episomal expression, either the empty vector (mock) or carrying the genes of interest, were transfected into L. infantum WT and mrpA C−/− promastigotes by nucleofection, as previously described (Fernandez-Prada et al., 2018). Selection was achieved in the presence of 300 μg/mL hygromycin.

2.3. Mutant selection

Five L. infantum puro/puro mrpA C−/− Sb-resistant mutants (mrpA C−/−/CL1 to mrpA C−/−/CL5) were independently selected from mrpA C−/− in 25 cm2 flasks containing 5 mL SDM-79 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 5 μg/mL hemin 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 3 μM up to 180 μM of SbIII. Last-level mrpA C−/− Sb-resistant mutants were grown in the absence of drug pressure for 20 passages to revert resistance (rev).

2.4. Drug susceptibility assays

Antileishmanial values in promastigotes were determined by monitoring the growth of parasites after 72 h of incubation at 25 °C in the presence of increasing antimony concentrations, by measuring A600 using a Cytation 5 machine (BioTek, USA). EC50 against antimony was evaluated in vitro infections using a luc-expressing episomal vector (pSP1.2 luc chyga) and PMA-differentiated THP-1 cells as previously described (Fernandez-Prada et al., 2016). Briefly, PMA-differentiated THP-1 macrophages were infected with stationary-phase parasites at a ratio of 18:1, for 2 h at 37 °C in a 5% CO2 atmosphere. Cells were maintained in drug-free medium for 48 h, after which infected cells were either left untreated or were treated with increasing concentrations of SbIII (Sodium Stibogluconate, Calbiochem) for 96 h at 37 °C. At this point, THP-1 cells were washed, and luciferase activity was determined using a Cytation 5. Drug-efficacy assays for both promastigotes and macrophage-infecting amastigotes were performed with at least three biological replicates from independent cultures (n = 3). EC50 values were calculated based on dose-response curves analysed by non-linear regression with GraphPad Prism 8.0 software (GraphPad Software, La Jolla California, USA). An average of at least three independent biological replicates was performed for each determination. Statistical analyses were performed using unpaired two-tailed t-tests. A p value < 0.05 was considered statistically significant.
2.5. Whole genome sequencing

Genomic DNAs were prepared from a mid-log phase clonal culture of each mutant. Fifty nanograms of purified gDNA were used for paired-end library preparation using Nextera™ DNA Sample preparation kit (Illumina) according to the manufacturer’s instructions. The size distribution of Nextera libraries was validated using an Agilent 2100 Bioanalyzer and High Sensitivity DNA chips (Agilent Technologies). Sequencing libraries were quantified with the QuantiFluor® dsDNA System and sequenced using an Illumina HiSeq system at a concentration of 8 pM. An average genome coverage of over 50-fold was achieved for each L. infantum mrpA / Sb-resistant mutant. This approach allowed identification of point mutations when compared with the reference genome sequence of L. infantum JPCM5 (TrizTrypDB version 8.0) (Aslett et al., 2010) and L. infantum 263 WT (Ritt et al., 2013). Sequence reads were aligned to the L. infantum JPCM5 genome using the software bwa-mem (El Fadili et al., 2009). The maximum number of mismatches was 4, the seed length was 32, and 2 mismatches were allowed within the seed. Read duplicates were marked using Picard (http://broadinstitute.github.io/picard), and GATK was applied for indel realignment and SNP and InDel discovery (McKenna et al., 2010; DePristo et al., 2011) in L. infantum mrpA / Sb-resistant mutants. PCR amplification and conventional DNA sequencing verified all putative point mutations detected by whole genome sequencing. Copy number variations (CNVs) were derived from read depth coverage by comparing the coverage of uniquely mapped reads between L. infantum mrpA / Sb-resistant mutants and L. infantum 263 WT in small non-overlapping genomic windows (5 kb) for the 36 chromosomes (normalized to the total number of uniquely-mapped reads for each strain) (Chiang et al., 2009). Several python and bash scripts were created to further analyse the data. The sequence data for L. infantum mrpA / Sb-resistant mutants is available at the NCBI BioProject (https://www.ncbi.nlm.nih.gov/bioproject/) under study accession PRJNA599612 and sample accessions SAMN13762311, SAMN13762312, SAMN13762313, SAMN13762314, SAMN13762315, SAMN13762316, SAMN13762317, SAMN13762318, SAMN13762319, SAMN13762320, corresponding to mrpA / Sb-resistant CL1 20 ×, CL2 20 ×, CL3 20 ×, CL4 20 ×, CL5 20 ×, CL6 20 ×, CL7 60 ×, CL8 60 ×, CL9 60 ×, CL4 60 × and CL5 60 ×, respectively.

2.6. PFGE and southern blot

Molecular karyotype was obtained from L. infantum WT, mrpA / Sb-resistant mutants, and revertants by separation of chromosomes through pulse field electrophoresis. 10⁸ mid-log phase parasites were embedded in low melting point agarose blocks, digested with proteinase K, and electrophoresed in a contour-clamped homogeneous electric field apparatus (CHEF Mapper, Bio-Rad, Hercules, CA, USA). The blocks were mounted in 1% agarose gel and electrophoresed in 0.5x Tris-Borate-EDTA running buffer at 5 V cm⁻¹ with a 120° separation angle at 14 °C for 30 h. A range of 150–1500 kb was applied for a wide chromosomal separation, resolving most Leishmania chromosomes in a single molecular karyotype gel. Saccharomyces cerevisiae chromosomes were used as a DNA size marker (Bio-Rad, Hercules, CA, USA). For Southern blots, genomic DNA was isolated using DNAzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions, then digested with the Ascl restriction enzyme (New England Biolabs Inc, Ipswich, MA, USA). Digested genomic DNA or

![Image](https://example.com/image.png)
3. Results

3.1. mrpA is not essential for L. infantum survival or infectivity but is key to modulation of parasite antimony sensitivity

The gene coding for the puromycin acetyltransferase (puro) was cloned between the 5′- and 3′- L. infantum mrpA flanking regions and the linear PURO construct was transfected by electroporation. Instead of using a second selectable marker for substituting the second mrpA allele, we decided to explore the possibility of a single round of targeted gene replacement followed by selection for heterozygosity (Gueiros-Filho and Beverley, 1996) by increasing by 2.5 fold the puromycin-selection pressure. As depicted in Fig. 1A, hybridization with a 5′UTR probe should lead to 6.5 and 2.4 kb AscI-AscI bands in the WT and puro/puro mrpA+/− parasites, respectively. As demonstrated in Fig. 1B (lane 2), we were able to generate a homozygous puro/puro mrpA+/− line, as no signal for the WT mrpA allele at 6.5 kb was detected for the mutant. This result was further confirmed by whole-genome sequencing of the mrpA−/− strain, as well as two PCR experiments (Fig. 1C): one targeting 200 bp within the gene (aa′) and another targeting the whole mrpA ORF using +30 nucleotide external primers (bb′). Once the mrpA−/− genotype was confirmed, puromycin-selection was not further continued.

Experimental dose-response assays with the mrpA−/− strain revealed an antimony hypersensitive phenotype in both promastigotes (Fig. 1D left panel; 20-fold Sbμ EC50 shift (P ≤ 0.001)) and macrophage-infecting amastigotes (Fig. 1D right panel; 2.6-fold Sbμ EC50 shift (P ≤ 0.001)). Transfection of the mrpA gene as part of an episomal plasmid was not further continued.

Selection of MRPA-independent Sb-resistant mutants began at 1 × EC50 value for the mrpA−/− strain (3.2 μM) up to 60 × EC50 (180 μM), which is approximately 2.5-fold the EC50 of the WT parental line. The selection procedure was fast (3 subsequent passages per drug concentration), and cells rapidly adapted to growing concentrations of Sb. All five clones reached the final selection step at 60 × EC50. In order to follow the evolution of the resistant phenotype for each clone, dose-response assays were conducted at passage 3 (P3) for 1 ×, 4 ×, 10 ×, 20 × and 60 × EC50 selection steps. As depicted in Fig. 2, all five clones reached similar EC50 values at selection step P3 10 × EC50, with clones CL1 and CL2 displaying slightly higher values (23 and 20 μM, respectively). While these two clones showed a great increase in EC50 values at selection step P3 20 × EC50 (48 and 63 μM, respectively), clones CL3, CL4 and CL5 remained with values around 20 μM. Once the final selection step was reached (P3 60 × EC50), clones were reevaluated for sensitivity against antimony. Clones CL1 and CL2 demonstrated the highest EC50 values (105 and 141 μM), higher than the EC50 value for the parental WT strain (approximately 65 μM). The other clones reached modest but significant increases in their EC50 values (CL3: 35; CL4: 48; and CL5: 38 μM), which represent a 6-fold mean increase compared to the unselected parental mrpA−/− strain. Potentially different mechanisms of adaptation to Sb are being deployed by independent clones.

3.3. Exposure of mrpA+/− mutants to increasing concentrations of Sb results in major changes in CNVs

Whole-genome sequencing was conducted by Illumina next-generation sequencing on the five independent L. infantum mrpA+/− Sb-resistant lines selected at 20 × and 60 × EC50, as well as the isogenic L. infantum WT line. For all strains, this produced genome assemblies of 31 Mb with a coverage depth of at least 50-fold.

Several cases of supernumerary chromosomes were observed in the mrpA−/− Sb-resistant mutants (Fig. 3 and Dataset S1), the majority of which had log2 mrpA−/− Sb-resistant/WT read ratios close to 0.5. Parasites from the Leishmania Leishmania subgenus have predominantly disomic genomes, and this should thus represent a gain of one allele compared to WT parasites (going from 2 to 3 chromosome copies). Most supernumerary chromosomes were shared by the mutants; chromosomes 6 and 9 were consistently increased in all mrpA−/− Sb-resistant mutants except CL4, chromosomes 18 (except in CL5 at 60 ×) and 23 were increased in all five mutants. Markedly, chromosome 23 passed from disomic to tetrasomic for CL5. Chromosome 8 was increased for clones CL1, CL3 and CL4; and chromosome 11 for clones CL1, CL4 and CL5. Finally, CL1, CL2 and CL4 gained a copy of chromosome 12, thus becoming pentasomic. There were no major differences between genome sequences at 20 × and 60 ×, with the exception of several chromosome losses, which were consistent with the loss of one allele (Fig. 3, Dataset S1). These losses affected chromosome 31 in CL2, CL3 and CL5. Overall, CL3 was the most divergent clone and displayed the highest level of chromosome-level CNVs compared to the four other mutants (Fig. 3). However, this did not correlate with reduced Sb sensitivity.

Normalized read depth coverage allowed the identification of amplified and deleted genomic loci in the mrpA−/− Sb-resistant mutants. These are characterized by punctuated series of adjacent genomic windows whose normalized read coverage varies compared to the WT baseline, as observed for chromosome 23 in more than one mutant (Dataset S2). In fact, for chromosome 23, the amplified region was large, covering 110 kb and 75 kb in mutants CL2 and CL5, respectively (Fig. 4A and B). This amplification starts at one subtelomeric end and encompasses the region where the mrpA gene was located before generation of the null mutant. This subtelomeric amplification was suspected to be a linear ampiclon rather than an intrachromosomal tandem duplication due to its length, its location close to a telomere, and the increase in number of normalized reads. Beside the amplification, normalized read depth coverage was higher in the mutant for the entire length of the chromosome (Fig. 4B) and supported the increase in ploidy described above for chromosome 23 (Fig. 3). Markedly, while the change of ploidy for all five clones seems to have been achieved at 20 × (Fig. 4A), the 60 × selection step was required to induce the subtelomeric amplification observed for clones CL2 and CL5 (Fig. 4B).

Hybridization of chromosomes separated by PFGE with probe-targeting gene LinJ.23.0220 (located nearby mrpA on chromosome 23) supported the NGS data and confirmed that two mutants had linear amplifications corresponding to the subtelomeric region of the chromosome (Fig. 4C, lanes 4 and 7). Regarding clone CL1, normalized read depth coverage of chromosome 23 revealed 2 peaks, which are also comprised in the subtelomeric amplifications observed for CL2 and CL5 (Fig. 4B). The first corresponds to the genomic region LinJ.23:65000-80000, which contains the partial ORF of LinJ.23.0230 (ABCC1) and the complete sequence of LinJ.23.0240 (ABCC2) (see chromosome 23 in Dataset S2); the second corresponds to mrpA gene-flanking regions, an artifact due to a duplication of the puro selectable marker within the 5′ and 3′ UTRs of mrpA.

3.4. Link between gene amplification and drug resistance

To verify if linear DNA amplifications were reversible, CL2 and CL5 (as well as the other clones) were subcultured in the absence of Sb
pressure for 20 passages. The chromosomes of the revertants of CL2 and CL5 were separated by PFGE with a probe targeting the gene LinJ.23.0220. Both clones lost their respective linear amplicon after 20 passages in the absence of Sb pressure (lanes 5 and 8 of Fig. 4C).

Next, the potential resensitization of clones after 20 passages in the absence of Sb pressure was evaluated phenotypically. As depicted in Fig. 4D, the EC50 values for all five clones were significantly reduced compared with their mrpA+/− Sb-resistant counterparts. CL1 and CL2 showed the greatest shift in EC50 values with 3- and 5-fold decreases, respectively. Shifts for clones CL3, CL4 and CL5 were more modest but still significant. None of the revertants reached the levels of hypersensitivity demonstrated by the parental mrpA+/− strain before stepwise selection.

We looked for genes that were both part of the linear amplicons identified in clones CL2 and CL5, and were present in the genomic region enriched in CL1 (LinJ.23:65000-80000), and noticed the presence of ABCC1 and ABCC2 genes (Leprohon et al., 2006). Overexpression of ABCC1 and ABCC2 was performed by stable episonal transfection in the mrpA−/− background, but also in WT parasites (Fig. 5A). While ABCC1 did not yield a resistant phenotype, overexpression of ABCC2 caused a 2-fold reduction in Sb sensitivity in the mrpA−/− strain. No effect was observed in the WT background for either ABCC1 or ABCC2 (Fig. 5A).

3.5. Point mutations and role of a serine acetyltransferase in antimony resistance

A search for point mutations revealed very few homozygous SNPs in any of the five mrpA−/− Sb-resistant mutants (Dataset S3), and none within any known drug resistance-related ORF. Homozygous SNPs were located in genes coding for hypothetical proteins, with the exception of LinJ.35.0500, which codes for a large proteophosphoglycan protein often found mutated in our various sequencing screens. Genes coding for hypothetical proteins were not studied further, as there was no recurrence among mutants for these genes. Heterozygous SNPs were more frequent, so they were clustered according to the number of clones carrying mutations in the same ORF (Supplementary Figs. S1 and S2; Dataset S3). Eight heterozygous SNPs were common to the 5 clones at both 20 × and 60 × . In all cases, exactly the same mutation was present. Given the high probability that our laboratory strain already carries these SNPs (absent in the reference strain), they were not further analysed. No SNPs were found by NGS in the aqp1 gene, which, when mutated or deleted, leads to a reduction in antimony uptake by the parasite (Monte-Neto et al., 2015). This was further confirmed by targeted PCR and sequencing of the aqp1 ORF from all five clones at both 20 × and 60 × selection steps. In order to find potential SNP candidates, the different clones were examined for SNPs occurring in the same ORF but at different positions. Only one SNP located in gene LinJ.34.2710 (serine acetyltransferase sat gene) in clones CL1, CL2 and CL4 at the 60 × selection step fulfilled this requirement (Supplementary Fig. S2). The mutations identified in the three mrpA+/− Sb-resistant mutants by NGS and confirmed by targeted PCR were satG325R for CL1, satG321R for CL2 and satG325R for CL4. Interestingly, the heterozygous SNPs in the sat ORF for CL1, CL2 and CL4 were lost from the populations of the clones when grown for 20 passages in the absence of antimony.

We overexpressed the three sat heterozygous mutations (as well as the WT version of the gene) in both mrpA+/− (Fig. 5B) and WT backgrounds (Fig. 5C). Overexpression of the satWT copy did not confer further Sb resistance in the mrpA−/− strain nor in the WT cell line. However, overexpression of all three mutated variants of the sat gene resulted in a significant decrease in Sb sensitivity in the mrpA−/− strain: satG325R (2.12-fold), satG321R (2.94-fold) and satG325R (3.23-fold). That said, only two of these three mutations, satG321R and satG325R, had a significant effect on Sb susceptibility in the WT strain (1.62- and 1.99-fold, respectively).

4. Discussion

Although the mode of action of Sb remains unclear, several mechanisms of drug resistance have been extensively described in Leishmania parasites, including modulation of Sb detoxification pathways (Mukherjee et al., 2007; Wylie et al., 2004), altered drug internalization (Monte-Neto et al., 2015; Marquis et al., 2005), and increased efflux (Mukhopadhyay et al., 1996). However, the most frequently reported mechanism of Sb resistance is the intracellular self-questration of Sb-thiol conjugates, mediated by ABC-transporter MRPA (Gazanion et al., 2016; Ouellelet and Papadopoulou, 1993; Leprohon et al., 2015; Ponte-Sucre et al., 2017). In this study, in order to search for MRPA-independent mechanisms of drug resistance against Sb, we utilized a unique combination of targeted disruption of the mrpA gene, stepwise drug-resistance selection, and next-generation sequencing (NGS) in L. infantum parasites.

Firstly, we demonstrated that MRPA is not essential for the survival and infectivity of L. infantum in vitro. As suspected, sensitivity to Sb was greatly increased in the mrpA−/− mutant. MRPA-deficient promastigotes demonstrated a 20-fold decrease in their EC50 value against Sb50. That said, mrpA−/− amastigotes showed a modest 2.6-fold increase in sensitivity against Sb50. Of note, although mrpA is frequently found in promastigote drug screens, and validated as a drug resistance gene with...
intracellular parasites (El Fadili et al., 2005), its role as an in vivo marker of Sb resistance is controversial since it’s not always found to be amplified in drug-resistant clinical isolates (Mukherjee et al., 2007; Moreira et al., 1998; Barrera et al., 2017) but increased in mrpA CNVs was found to be the main driver of molecular preadaptation to antimony resistance in L. donovani (Dumetz et al., 2018). Interestingly, two different gain-of-function screens using genomic overexpression libraries coupled to NGS (Cos-Seq) revealed a very pronounced enrichment of the mrpA gene in promastigotes progressively exposed to SbIII, but not in macrophage-infecting amastigotes exposed to SbV (Gazanion et al., 2016; Fernandez-Prada et al., 2018), showing that MRPA-independent Sb-resistance mechanisms can be deployed by the parasite.

In order to identify novel MRPA-independent Sb-resistance

Fig. 3. Dynamics of aneuploidy of L. infantum mrpA−/− during in vitro adaptation to antimony. Heatmap representation of log2-transformed normalized Sb-resistant/unselected read ratio for all 36 chromosomes in the five independent L. infantum mrpA−/− Sb-resistant lines selected at 20 × and 60 × EC50. Chromosomes were divided into non-overlapping 5 kb genomic windows and median L. infantum mrpA−/− Sb-resistant/unselected reads ratios for each chromosome were normalized according to the total number of reads followed by log2-transformation (See Dataset S1 for further details).

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mechanisms, we evaluated the ability of five independent *L. infantum mrpA*~−/−~ clones to become resistant to SbIII. In this study, all five clones were able to significantly decrease their sensitivity to Sb in comparison to the parental *L. infantum mrpA*~−/−~ line, but only two clones (CL1 and CL2) were able to surpass the EC50 value calculated for the parental WT strain. These results point to different genomic adaptation mechanisms occurring in each of the five independent clones. As gene expression in *Leishmania* is regulated predominantly by gene dosage (Leprohon et al., 2015; Iantorno et al., 2017), we proceeded to identify CNVs and SNP events in the five clones at two different selection steps, 20 × and 60 × EC50 of the unselected *L. infantum mrpA*~−/−~ strain, by NGS (Leprohon et al., 2015). Normalized read depth coverage identified several chromosomes in the *mrpA*~−/−~ Sb-resistant clones whose ploidy was altered compared to the unselected parental line. However, no common pattern among karyotypes was found (except for chromosomes 18, 23 and 31, which displayed common changes in CNVs for almost all the clones), which makes it difficult to establish a link between phenotypes and karyotype variations. This is not new as *Leishmania* chromosome ploidy in individual parasites is known to vary rapidly in response to changing environments, including routine cell culture, resulting in mosaic aneuploidy (Sterkers et al., 2011; Ubeda et al., 2008). A recent study identified major changes in chromosomal somy, gene expression and gene dosage driven by SbIII in *L. braziliensis* (Patino et al., 2019). Markedly, our results for chromosomes 4 (CL1 and CL3), 11 (CL1, CL4 and CL5), 14 (CL2 and CL3) and 23 (all the clones) are in line with those reported by Patino and co-workers (Patino et al., 2019).

*Leishmania* accumulates SbIII primarily through aquaglyceroporin AQP1, which is involved in volume regulation and osmotaxis (Gourbal et al., 2004). Loss-of-function mutations in its ORF, as well as CNVs at the level of the subtelomeric region of chromosome 31 harboring the *aqp1* gene result in decreased SbIII susceptibility (Monte-Neto et al., 2015; Mukhopadhyay et al., 2011; Plourde et al., 2015; Imamura et al., 2016). Sb-selection did not induce mutations in the *aqp1* gene in any of our *mrpA*~−/−~ Sb-resistant clones. However, CL2, CL3, and CL5 reduced the ploidy of chromosome 31 by one copy at 60 × EC50, which could result in decreased Sb uptake. This finding corroborates previous studies demonstrating that *Leishmania* parasites submitted to experimental Sb selection rely on aneuploidy as their first adaptation mechanism, even before emergence and eventual fixation of SNPs (Mukherjee et al., 2013; Monte-Neto et al., 2015; do Monte-Neto et al., 2011).

Alternatively, chromosome 23, harboring the H locus in which *mrpA*...
is located (Leprohon et al., 2009a), was amplified in all five of our mrpA−/− Sb-resistant clones, transitioning from a disomic to trisomic ploidy in CL1 to 4, and to a tetrasomic ploidy in CL5. Markedly, the subtelomeric region of chromosome 23 was further amplified in CL2 and CL5 in the form of two different linear amplicons. On the other hand, CL1 demonstrated major enrichment of a 15-kb genomic region comprising the partial sequence of LinJ.23.0230 (ABCC1) and the complete ORF of LinJ.23.0240 (ABCC2), which is also included in the linear amplicons detected for CL2 and CL5. While it has been shown that mrpA is the only gene within the H locus that contributes to Sb resistance when overexpressed (Dumetz et al., 2018; Leprohon et al., 2009b; Callahan and Beverley, 1991), we decided to evaluate the impact of ABC1/ABC2 overexpression and different mutations in the sat gene in Sb susceptibility. (A) Impact of overexpression of the genes ABC1 and ABC2 on the Sb-sensitivity profile of both WT and mrpA−/− lines. (B) Impact of overexpression of the sat gene, as well as three mutated versions, on the Sb-sensitivity profile of mrpA−/− parasites. (C) Impact of overexpression of the sat gene and its three mutated versions on the Sb-sensitivity profile of WT parasites. Data are the mean ± SD of three biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

Fig. 5. Functional analysis of ABC1/ABC2 overexpression and different mutations in the sat gene in Sb susceptibility. (A) Impact of overexpression of the genes ABC1 and ABC2 on the Sb-sensitivity profile of both WT and mrpA−/− lines. (B) Impact of overexpression of the sat gene, as well as three mutated versions, on the Sb-sensitivity profile of mrpA−/− parasites. (C) Impact of overexpression of the sat gene and its three mutated versions on the Sb-sensitivity profile of WT parasites. Data are the mean ± SD of three biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).
trypanothione and glutathione) through, by example, disabling of a regulatory site or negative feedback loop limiting such products, future work should evaluate the content of the different thiol species in the mrpA−/− mutant background as well as in the Sb-resistant mrpA−/− clones and establish whether this correlates with specific changes in sensitivity to Sb.

Amplification of MRPA or loss-of-function mutations in AQP1 are the primary mechanisms of Sb resistance in both laboratory and field isolates (Mukherjee et al., 2007; Dumetz et al., 2018; Imamura et al., 2016). That said, these strong traits could preclude the identification of secondary mechanisms/markers of drug resistance against Sb. In order to evaluate the occurrence of SNPs in the sat gene in clinical isolates, the TritrpyDB database (Aslett et al., 2010) was mined for genetic variations in the sat gene among its L. donovani dataset, which comprise a total of 252 isolates. Six non-synonymous polymorphisms were found (lines 234,631 to 264,639 highlighted in yellow in the Ldo-no_allTritrpy_SNPS tab of Dataset 4). The strains harboring the changes were retrieved from a nucleotide alignment of the sat gene for the 252 isolates (tabs strains Dataset 4). Most of the strains including SNPs in the sat gene are associated with a dataset derived from a study of Ethiopian L. donovani (Zackay et al., 2018). However, Zackay and co-workers only explored the drug-resistant profile of these strains against methotrexate because they were able to identify CNVs for the folate uptake and modulation of drug resistance in parasites. Eukaryot. Cell 5 (10), 1713–1725.

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