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A single amino acid substitution (H451Y) in Leishmania calcium-dependent kinase SCAMK confers high tolerance and resistance to antimony

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1 **Title: A single amino acid substitution (H451Y) in *Leishmania* calcium-dependent kinase**
2 **SCAMK confers high tolerance and resistance to antimony.**

3 **Running title:** *Leishmania* tolerance and resistance

4

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18 **Synopsis:**

19 **Background:** For almost a century, antimonials remain the first-line drugs for the treatment
20 of leishmaniasis. However, little is known about their mode of action and clinical resistance
21 mechanisms.

22 **Objectives:** We have previously shown that *Leishmania* nicotinamidase (PNC1) is an essential
23 enzyme for parasite NAD⁺ homeostasis and virulence *in vivo*. Here, we found that parasites
24 lacking *pnc1* gene ($\Delta pnc1$) are hypersusceptible to the active form of antimony (SbIII) and used
25 these mutant parasites to better understand antimony mode of action and resistance.

26 **Methods:** SbIII-resistant WT and $\Delta pnc1$ parasites were selected *in vitro* by stepwise selection
27 method. NAD(H)/NADP(H) dosages and quantitative RT-PCR experiments were performed to
28 explain the susceptibility differences observed between strains. WGS and a marker-free
29 CRISPR/Cas9 base editing approach were used to identify and validate the role of a new
30 resistance mutation.

31 **Results:** NAD⁺ depleted $\Delta pnc1$ parasites are highly susceptible to SbIII and this phenotype can
32 be rescued by NAD⁺ precursor of trypanothione precursors supplementation. $\Delta pnc1$ parasites
33 can become resistant to SbIII by unknown mechanism. WGS revealed a unique amino acid
34 substitution (H451Y) in an EF-hand domain of an orphan calcium-dependent kinase recently
35 named SCAMK. When introduced into a wild type reference strain by base editing, the H451Y
36 mutation allows *Leishmania* parasites to survive at extreme concentrations of SbIII,
37 potentiating the rapid emergence of resistant parasites.

38 **Conclusions:** These results establish that *Leishmania* SCAMK is a new central hub of antimony
39 mode of action and resistance development and uncover the importance of drug tolerance
40 mutations in the evolution of parasite drug resistance.

41 Introduction

42 *Leishmania* are protozoan parasites transmitted by sandflies that are responsible for a wide
43 spectrum of human infections ranging from the life-threatening visceral disease to disfiguring
44 mucosal and cutaneous forms¹. Treatment of leishmaniasis is limited to four main drugs
45 (antimonials, miltefosine, amphotericin B and paromomycin). Alone or in combination,
46 antimonials have been the mainstay of anti-*Leishmania* therapy worldwide for over 70 years.
47 However, our knowledge of antimony mode of action is still partial and the emergence of
48 parasite resistance to antimony in the Indian subcontinent during the last decades has
49 necessitated the use of alternative medications. The active form of antimony (SbIII) is known
50 to target the *Leishmania* redox potential by inducing rapid thiols efflux and inhibiting the
51 NADPH-dependent trypanothione reductase (TR) that maintains the main parasite thiol,
52 trypanothione, in its reduced form² (Figure 1a). *Leishmania* resistance to SbIII is generally
53 mediated by drug uptake reduction through the aquaglyceroprotein 1 (AQP1) transporter
54 mutations or down-expression, or by drug efflux/sequestration through the concomitant
55 overexpression of genes involved in the parasite thiol metabolism and of the gene encoding
56 the ATP-binding cassette transporter ABC-C3 (MRPA)^{3,4} (Figure 1a). Other transporters of the
57 ABC superfamily also interfere with antimonials drug accumulation, including ABC-C7 (PRP1)⁵,
58 ABC-I3⁶, ABC-I4⁷ or ABC-G2⁸. In addition, it has been shown that a number of intracellular
59 proteins can also modulate the susceptibility of parasites to SbIII when they are overexpressed
60 in experimental strains or clinical isolates. These include heat shock proteins (Hsp23⁹,
61 Hsp83¹⁰), kinase (MAPK1¹¹), serine/threonine phosphatase (LinJ.12.0610¹²) or proteins with
62 still unknown functions (ARM56⁹, ARM58⁹, P299¹³). Overall, these results illustrate that
63 *Leishmania* parasites have multiple strategies to acquire drug resistance and highlight the
64 difficulty to define a conserved molecular marker of antimony resistance.

65 In a previous work¹⁴, we showed that *Leishmania* parasites are auxotrophic for NAD⁺
66 and rely on a salvage pathway to recycle NAD⁺ precursors from their environment (Figure 1a).
67 The nicotinamidase PNC1 is a central enzyme of this pathway that controls NAD⁺ homeostasis
68 by hydrolysing nicotinamide (NA_m) to nicotinic acid (NA). *Leishmania infantum* parasites in
69 which the *pnc1* gene was disrupted ($\Delta pnc1$) are depleted in NAD⁺ and cannot cause durable
70 infections in mice, making of this enzyme an attractive target for drug development^{14,15}.
71 NAD(H), and its derived phosphorylated forms NADP(H), are essential cofactors required for

72 energy-producing pathways and antioxidant defence in all living cells. They are further key
73 metabolites involved in host-pathogen interactions¹⁶. Here, we used NAD⁺ depleted $\Delta pnc1$
74 mutant parasites to better understand SbIII mode of action and the mechanisms leading to
75 resistance to this drug. We found that intracellular NAD⁺ levels can directly modulate the
76 susceptibility of parasites to SbIII and identified the first mutation conferring antimony
77 tolerance and resistance in *Leishmania*.

78 **Materials and Methods**

79 ***Strains and cultures***

80 The *pnc1* null mutant ($\Delta pnc1$) was previously generated by targeted gene replacement in the
81 *L. infantum* (MHOM/MA/67/ITMAP-263) strain¹⁴. CRISPR/Cas9 genome edition experiments
82 were performed in the *L. major* “Friedlin” reference strain (MHOM/IL/80/Friedlin,
83 LEM3171)¹⁷. All strains and the derived mutants were maintained as promastigote forms in
84 SDM79 medium supplemented with 10% FBS, penicillin/streptomycin and hemin (5 mg/L)
85 (complete medium). Potassium antimonyl tartrate trihydrate (SbIII), L-Glutathione reduced
86 (GSH), N-Acetyl-L-cysteine (NAC), nicotinic acid (NA) and MTT were all purchased from Sigma-
87 Aldrich. SbIII-resistant parasites were generated by a stepwise approach starting with a drug
88 concentration corresponding to the EC₅₀ for that strain. Growth curve and EC₅₀ were
89 determined starting from an inoculum of 10⁶ parasites/mL in 5 mL of complete medium,
90 unless otherwise stated. Parasite density was determined daily (growth kinetics) or after 3
91 days of incubation with the drug (EC₅₀ determination) using a flow cytometer¹⁴ or by
92 measuring the OD at 600 nm (for graphical convenience, OD values were multiplied by 1000).
93 All EC₅₀ values were obtained with GraphPad Prism v5 software using a sigmoidal dose-
94 response model with variable slope. Cell viability assays on edited parasites were performed
95 using a MTT test. In this assay, the yellow tetrazolium MTT dye was reduced to insoluble
96 formazan crystals (purple color) in living cells using NADH as reducing agent. Briefly, 100 μ L of
97 parasite cultures were mixed with 10 μ L of a MTT solution (10 mg/mL in PBS) and incubated
98 4h at 26°C. The reaction was stopped with 100 μ L of lysis solution (50% isopropanol/10% SDS)
99 and the plates were incubated for an additional 30 min with gentle shaking in the dark. The
100 change in colour from yellow to purple was read at an absorbance of 600 nm.

101 ***NAD(H)/NADP(H) quantification***

102 Total (NAD⁺ plus NADH) or individual (NAD⁺ and NADH) dinucleotides were quantified using
103 the bioluminescent NAD/NADH-Glo™ Assay (Promega) following manufacturer’s instructions.
104 This kit uses a proluciferin substrate to produce a light signal proportional to the amount of
105 NAD and/or NADH present in the samples. Total (NADP⁺ plus NADPH) and individual (NADP⁺
106 and NADPH) phosphorylated forms were quantified similarly using the NADP/NADPH-Glo™

107 Assay kit (Promega). Data are expressed as the mean value (\pm SD) of Relative Luminescence
108 Units (RLU) obtained for 10^6 parasites and after 60 min of incubation.

109 **Quantitative RT-PCR analyses (qRT-PCR)**

110 All primers used in this study were designed with the primer3Plus software and are listed in
111 table S1. Total RNA was extracted from parasite cultures in the exponential phase of growth
112 using the RNeasy+ Mini Kit (Qiagen) with an additional treatment with turbo DNA-free DNase
113 (Thermo Fischer Scientific). Two μ g of total RNA was transcribed into complementary DNA
114 (cDNA) using the Superscript reverse transcriptase III (Invitrogen) and oligo(dT)12-18 primers
115 (Thermo Fischer Scientific), according to the manufacturer's instructions. Diluted cDNA (1/10)
116 was used for the qPCR reactions in 10 μ L final volume with SYBR Green I Master on a
117 LightCycler480 (Roche). The relative expression of each gene was determined from two
118 biological replicates with the $2^{-\Delta\Delta CT}$ method and the *GAPDH* gene as reference, using the
119 LightCycler480 software.

120 **CRISPR/Cas9 genome editing and off-target mutation analysis**

121 The marker-free nucleotide editing approach developed in *P. falciparum*¹⁸ was adapted to
122 introduce the H451Y mutation in the *L. major* reference strain. The 20nt single guide RNA
123 (sgRNA) sequence TTTGATCGACTCCGAGCACT (the targeted nucleotide is underlined) was
124 based on the *LmjF.33.1710* orthologue gene sequence. Plasmids were constructed as
125 described in¹⁷. Briefly, a donor DNA sequence that consists of an 880 bp-long intragenic region
126 of *LmjF.33.1710* gene was generated by PCR fusion using the modified primer pairs
127 Lm1F/Lm1R and Lm2F/Lm2R (Table S1) to create the C1351T mutation and a "shield" silent
128 mutation (G1356T) in the protospacer adjacent motif (PAM) to prevent further Cas9-mediated
129 cleavage (Figure 2f). The donor DNA sequence was cloned in the pLS5 plasmid digested with
130 *KpnI* and *XbaI*. To obtain the 20nt sgRNA sequence surrounded by the 15nt adaptors necessary
131 for In-Fusion[®] cloning, the two oligonucleotides Seed_F and Seed_R were annealed by
132 incubation in boiling water for 2min followed by gentle cooling in room-temperature water
133 for 2h. The sgRNA sequence and adaptors were then cloned in the *BsgI*-digested pLS5-donor
134 DNA plasmid using the In-Fusion HD cloning kit (Clontech). pLS5-donor DNA plasmids with
135 and without the sgRNA sequence were transfected in the *L. major* Friedlin strain that harbours
136 the pTCAS9 plasmid¹⁷ to generate edited (Lm_H451Y) and control (Lm_ctrl) cell lines,

137 respectively. After transfection and selection with hygromycin and puromycin, gene edition
138 was checked by PCR amplification and Sanger sequencing using the
139 LmiSCAMK_F/LmiSCAMK_R primer pair (Table S1). Once heterozygosity was confirmed in the
140 mutated locus, parasites were cloned by limiting dilution. A parasite clone containing the
141 homozygous desired and shield mutations (named Lm_H451Y) was selected for subsequent
142 phenotype analyses. To confirm the absence of off-target mutations, the genomes of Lm_ctrl
143 and Lm_H451Y strains were sequenced (see below) and off-target candidates (up to five
144 mismatches allowed) were identified using the Protospacer Workbench software suite¹⁹. For
145 each candidate, the vicinity (within a 21nt window) to specific INDELS of the edited cell lines
146 was assessed as described in Vasquez *et al.*²⁰ (Table S2).

147 **WGS and analyses**

148 Parasite genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). A paired-end
149 sequencing library for $\Delta pnc1$ parasites was prepared with the Nextera DNA Sample
150 Preparation Kit (Illumina) and sequenced on an Illumina HiSeq 2500 (2x250 bp) apparatus.
151 DNA samples from the KO-SbR, Lm_ctrl, Lm_H451Y and Lm_H451Y-SbR strains were sheared
152 using a Bioruptor Pico sonication device (Diagenode) to yield \approx 400 bp fragments. Libraries
153 were constructed for WGS as previously described²¹. Paired-end sequencing (2 x 150 bp) was
154 performed on an Illumina MiSeq apparatus with the MiSeq Reagent Kit V2. The presence of
155 SNPs and INDELS was determined using the EuPathDB Galaxy platform²² and the workflow
156 pipeline for “Variant Calling, paired-end sequencing” and pre-loaded reference genomes for
157 the *L. infantum* JPCM5 and *L. major* Friedlin strains (TritrypDB build 29). Filtered Variant Call
158 Format (VCF) files corresponding to SNPs and INDELS with an impact on coding sequences
159 were compared between the $\Delta pnc1$ and KO-SbR libraries. Specific SNPs/INDELS detected in
160 KO-SbR (16 SNPs and 15 INDELS) were manually verified with the IGV 2.4.8 visualization tool,
161 and eliminated if already present in the $\Delta pnc1$ parental line. After this verification step, only
162 three specific SNPs were retained for analysis (Table S3). The same strategy was used to
163 compare SNPs/INDELS in the Lm_ctrl, Lm_H451Y and Lm_H451Y-SbR libraries and only SNPs
164 and INDELS with read depth >2 were kept for further analysis (Table S4). The CNV-seq pipeline
165 was used to identify CNVs potentially associated with drug resistance²³. This pipeline identifies
166 localized regions in which the read depth normalized across the length of the chromosome
167 differs significantly between samples. For $\Delta pnc1$ and KO-SbR pairwise comparisons, a 3kb

168 window size was chosen, but similar results were obtained with smaller and larger window
169 sizes (Table S5). For Lm_H451Y and Lm_H451Y-SbR pairwise comparisons, the sliding window
170 size was automatically determined (4265 bp). Input hits files were derived from the Binary
171 Alignment Map (BAM) files generated by the EupathDB Variant Calling pipeline.

172 ***Accession number***

173 All sequencing data have been submitted to the European Nucleotide Archive (ENA) and are
174 available under the accession number PRJEB27329.

175 Results

176 *Δpnc1* parasites are highly susceptible to SbIII

177 When grown in SDM79 medium, *Δpnc1* promastigotes had similar growth rates than wild type
178 (WT) parasites (Figure 1b). However, they are about ten-fold more susceptible to SbIII (EC₅₀
179 *Δpnc1* = 2.51 μg/mL; EC₅₀ WT = 20.92 μg/mL) (Figure 1c). This phenotype could be rescued by
180 NA supplementation in the growth medium (Figure 1c). NA supplementation also induced a
181 dose-dependent increase of both NAD(H) and NADP(H) content in *Δpnc1* parasites where
182 these pools are significantly depleted (Figure 1d). Conversely, it did not have any effect on
183 NAD(H) and NADP(H) content in WT parasites (Figure 1d). Individual dinucleotides
184 measurements showed that *Δpnc1* parasites were specifically depleted in NAD⁺ and NADPH
185 forms (Figure 1e). As the *Leishmania* redox homeostasis is also maintained by *de novo*
186 biosynthesis of reduced trypanothione (Figure 1a), we tested whether supplementation with
187 trypanothione precursors could restore the physiological SbIII susceptibility in *Δpnc1*
188 parasites. Supplementation with N-acetyl-cysteine (NAC) or reduced glutathione (GSH)
189 decreased the susceptibility of *Δpnc1* parasites to SbIII (Figure 1f). Altogether, these results
190 established a direct connection between *Leishmania* NAD⁺ metabolism, SbIII toxicity and thiol
191 metabolism (Figure 1a). qRT-PCR assays further indicated that known genes involved in SbIII
192 mode of action and resistance were similarly transcribed in *Δpnc1* and WT parasites, except
193 for *GSH1*, the rate-limiting step in glutathione biosynthesis, for which the transcript level was
194 increased 2-fold in *Δpnc1* (Figure 1g).

195 *Δpnc1* parasites resistant to SbIII show a single amino acid substitution in the SCAMK gene

196 To better understand the link between NAD⁺ homeostasis and SbIII susceptibility, we
197 tried to generate SbIII-resistant WT and *Δpnc1* parasites (named WT-SbR and KO-SbR,
198 respectively) by stepwise selection in the presence of increasing concentrations of the drug.
199 We could select KO-SbR parasites that proliferated in the presence of up to 320 μg/mL of SbIII
200 (EC₅₀ = 309 μg/mL), but not at higher concentrations (Figure 2a). In WT-SbR parasites (EC₅₀
201 >1000 μg/mL), MRPA was ten-fold upregulated as compared with WT parasites (Figure 2b)
202 which is in agreement with the known model of antimony resistance^{3,4} and the presence of
203 an extrachromosomal amplicon of 15 kb bearing *MRPA* gene in the WT-SbR strain (data not
204 shown). Conversely, qRT-PCR analysis showed a modest, but paradoxical overexpression of

205 the genes encoding the AQP1 transporter and MRPA efflux-pump in KO-SbR parasites (Figure
206 2b). Moreover, the levels of NAD⁺, NADH, NADP⁺ and NADPH were similar in KO-SbR and
207 $\Delta pnc1$ parasites (Figure 2c). As drug resistance in *Leishmania* is often associated with gene
208 copy number variations (CNVs) or SNPs²⁴, we sequenced the genomes of $\Delta pnc1$ and KO-SbR
209 parasites by Illumina paired-end sequencing. As expected, no read aligned to the *pnc1* gene,
210 confirming its deletion in the both strains (Figure S1). All CNVs detected in KO-SbR mutants
211 were located in non-coding telomeric or intergenic regions (Table S5). The look for non-
212 synonymous SNPs only present in the KO-SbR strain identified a single homozygous mutation
213 (C1351T) leading to the H451Y substitution in a putative protein kinase gene (*LinJ.33.1810*)
214 (Table S3). The fixation of the H451Y mutation in KO-SbR parasites was confirmed by PCR and
215 Sanger sequencing (Figure 2d). Interestingly, another point mutation (E629K) in the C-
216 terminus of the *LinJ.33.1810* gene was already described in a *L. infantum* SbIII-resistant strain
217 bearing *MRPA* amplification²⁵. This single copy gene defines a new kinase family recently
218 named SCAMK²⁶ that is specific to Metakinetoplastina protists and functionally related to the
219 calcium-dependent protein kinases (CDPKs) present in plants and protozoans²⁷. CDPKs have
220 been widely studied in apicomplexan parasites where they are considered to be important
221 signal transducers involved in parasite motility, invasion or host cell egress²⁸. Specifically, Ca²⁺
222 binding to the EF-hand domains of CDPKs induces a global conformational change of the
223 protein, resulting in the activation of the kinase activity. The H residue in position 451, which
224 is mutated in the KO-SbR strain, is located within the calcium-binding site of the third EF-hand
225 domain of *Leishmania* SCAMK, and is conserved across kinetoplastids (Figure 2e).

226 ***The H451Y mutation generates SbIII tolerant Leishmania parasites***

227 To gain insight into the role of the H451Y mutation in SbIII resistance, we used the
228 CRISPR/cas9 genome editing technology¹⁷ with a marker-free nucleotide editing approach¹⁸
229 to introduce the H451Y mutation in the *Leishmania major* WT “Friedlin” reference strain. *L.*
230 *major* parasites expressing Cas9 from an episomal plasmid were transfected with a second
231 plasmid bearing an sgRNA complementary to the *L. major* SCAMK orthologue (*LmjF.33.1710*),
232 and a donor sequence including the desired mutation (C1351T) and a shield mutation
233 (G1356T) in the PAM motif (Lm_H451Y strain) (Figure 2f). As a control, we transfected
234 parasites with the same plasmid backbone, but without the sgRNA (Lm_ctrl strain) (Figure 2g).
235 After selection and cloning, we confirmed the presence of the H451Y mutation (Figure 2g) and

236 the absence of off-target mutations (Table S2) by Sanger and WGS, respectively. Phenotype
237 analyses indicated that Lm_ctrl and Lm_H451Y strains had similar growth rates (Figure 3a).
238 The SbIII susceptibility of both strains was compared using a MTT-based assay test and an
239 inoculum of 10^6 parasites/mL. As shown in figure 3b, Lm_H451Y parasites are not more
240 resistant than controls. Surprisingly, Lm_H451Y (but not Lm_ctrl) parasites exposed to the
241 highest drug concentrations resumed growth after seven days in culture (Figure 3c). Analysis
242 of the growth curves obtained starting from a higher parasite inoculum ($4 \cdot 10^6$ parasites/mL)
243 and using cytotoxic concentrations of SbIII showed that Lm_H451Y parasites can survive and
244 slowly proliferate in the presence of 1000 $\mu\text{g/mL}$ of SbIII, which is the limit of SbIII solubility in
245 the culture medium (Figure 3d). In a parallel experiment, the viability of edited parasites
246 exposed to maximal SbIII concentrations has been confirmed and quantified by MTT-based
247 assays performed at days 3 and 5 of culture (Figure 3e). Light-microscopy examination after
248 exposure to the maximal concentration of SbIII revealed the presence of a heterogeneous
249 population of dead and unstressed promastigote forms in Lm_H451Y cultures, and the
250 absence of surviving parasites in Lm_ctrl cultures (Figures 3f and S2). In agreement, we
251 detected intact high molecular weight genomic DNA in all tested conditions for Lm_H451Y
252 parasites, but only up to 250 $\mu\text{g/mL}$ of SbIII for Lm_ctrl parasites (Figure 3g). The ability of a
253 microorganism to survive lethal concentrations of a drug while keeping similar EC_{50} than
254 susceptible strains is characteristic of drug tolerant/persistent microorganisms²⁹. This
255 phenomenon is known to facilitate drug resistance acquisition in bacteria but remains largely
256 understudied in protozoan parasites³⁰. To confirm the role of the H451Y mutation in
257 *Leishmania* SbIII tolerance, we transiently exposed parasites to various bolus doses of SbIII for
258 two days, and followed their growth after drug withdrawal. The growth of Lm_ctrl parasites
259 was delayed and they could survive after transient exposure to SbIII concentrations up to 400
260 $\mu\text{g/mL}$ (Figure 3h). Conversely, Lm_H451Y parasites recovered within 10 days post-bolus,
261 whatever the concentration of SbIII used (Figure 3h). The toxicity of most anti-leishmanial
262 drugs has been partly attributed to induction of reactive oxygen species (ROS) formation and
263 consequently to oxidative damage. Therefore, we carried out similar growth curve
264 experiments in the presence of miltefosine, amphotericin B, the oxidative stress inducer
265 menadione, or H_2O_2 (Figure S3). We did not detect any difference between Lm_H451Y and
266 Lm_ctrl parasites in all tested conditions, suggesting that the tolerance phenotype induced by
267 the H451Y mutation is specific to SbIII mode of action and does not involve a generic response

268 to oxidative stress. Next, we tested whether Lm_H451Y parasites were prone to rapidly
269 develop resistance to SbIII. As expected, highly resistant Lm_H451Y parasites (named
270 Lm_H451Y-SbR) were selected after only five passages (P5) in the presence of 1000 µg/mL of
271 SbIII (EC₅₀ >1000 µg/mL) and this resistance phenotype remained stable up to P15 (Figure 3i).
272 Genome sequencing of Lm_H451Y-SbR parasites cultured for 10 passages in the presence of
273 1000 µg/mL of SbIII did not reveal any new CNV compared with the Lm_H451Y parental cell
274 line. Moreover, non-synonymous SNPs specific to Lm_H451Y-SbR were all heterozygous and
275 only concerned hypothetical proteins or factors not known to be involved in SbIII resistance
276 (Table S4). These results corroborate our initial observations in the *L. infantum* KO-SbR mutant
277 and show that the H451Y mutation is necessary and sufficient to generate SbIII-tolerant
278 parasites that can rapidly evolve into resistant parasites in the presence of drug pressure.

279 **Discussion**

280 Drug resistance in eukaryotic microorganisms shares many similarities with antibiotic
281 resistance in bacteria³. Our knowledge about drug resistance mechanisms in *Leishmania* (and
282 other protozoan parasites) mainly results from experiments using growth inhibitory
283 measurements that determine the lowest drug concentration needed to inhibit parasite
284 growth by 50% (EC₅₀). Mechanisms of drug action and resistance patterns can be however
285 different when measured at dosages that kill the parasite, not just inhibit its growth³¹.
286 Moreover, these approaches do not allow the identification of parasite subpopulations with
287 variable levels of resistance. In bacteria or fungi, drug tolerant subpopulations (also known as
288 “persisters”) can survive at lethal drug concentrations while being still sensitive to cytostatic
289 effect of the drug. The mechanisms leading to persisters formation are diverse and can include
290 genetic mutations that increase the proportion of persister cells within a population^{32,33}.
291 Importantly, persisters are known to cause treatment failure due to relapsing infections and
292 behave as an evolutionary reservoir of drug resistance^{30,34,35}. The phenomenon of drug
293 tolerance/persistence in protozoan parasites and its link with parasite drug resistance and
294 treatment failure have been poorly studied to date. In trypanosomatids, the ability of drug-
295 sensitive parasites to survive a lethal and prolonged drug exposure has been recently
296 described in *Trypanosoma cruzi*³⁶ and *Leishmania*³⁷. However, these phenotypes have been
297 attributed to clearly distinct mechanisms involving a metabolic dormancy state and a genetic
298 preadaptation to resistance, respectively.

299 In this study, we report that (i) *Leishmania* NAD⁺ depletion led to SbIII hypersusceptibility that
300 is rescued by NAD⁺ precursor or trypanothione precursors supplementation; (ii) NAD⁺
301 depleted parasites can develop SbIII resistance through a single amino acid substitution in a
302 metakinetoplastid-specific kinase; (iii) this mutation makes *Leishmania* parasites tolerant to
303 high concentrations of SbIII and facilitates the emergence of resistant parasites without
304 additional genomic modification. Although these findings have been obtained in mutants
305 selected under laboratory conditions *in vitro*, they provide novel insights into the mode of
306 action of antimony and establish that the orphan *Leishmania* kinase SCAMK is a new central
307 actor of SbIII mode of action and resistance. Because the H451Y mutation is precisely located
308 within the calcium-binding site of the third EF-hand domain of *Leishmania* SCAMK, we can
309 speculate that it should directly impair Ca²⁺ binding and the associated conformational change

310 required for the enzymatic activation of CDPKs. The study of the structural consequences of
311 the H451Y mutation should help us in understanding how this orphan kinase integrates
312 calcium signaling to control the lethal effect of SbIII and could result in new therapeutic
313 applications. We were unable to analyse the tolerance phenotype of edited parasites in the
314 intracellular amastigote stage because the *L. major* strain used in this study is a reference
315 strain that lost its virulence due to repeated passages *in vitro*. Further work is therefore
316 required to determine whether this mutation can lead to treatment failure and persistent
317 infection *in vivo* and to assess whether it is harmful or whether it can be spread in natural
318 populations. Nevertheless, the correlation between *Leishmania SCAMK* gene polymorphisms
319 and antimony treatment failure in clinical isolates could constitute the first step towards the
320 identification of a new molecular marker of antimony tolerance/resistance in the field.

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415 **Figure legends**

416 **Figure 1. $\Delta pnc1$ parasites are highly susceptible to SbIII. (a)** Schematic representation of the
417 cross-talk between *Leishmania* NAD⁺ metabolism, trivalent antimony (SbIII) mode of action
418 and thiol metabolism. NA, nicotinic acid; NAm, nicotinamide; NR, nicotinamide riboside;
419 NAMN, nicotinic acid mononucleotide; NAAD, nicotinic acid dinucleotide; NADK, NAD⁺ kinase;
420 PPP, pentose phosphate pathway; G6PDH, glucose-6-phosphate dehydrogenase; AQP1,
421 aquaglyceroporin 1; TR, trypanothione reductase; T(SH)₂, reduced trypanothione; T(S)₂,
422 oxidized trypanothione; TXN1, tryparedoxin 1; MRPA, ABCC3 transporter; Glu, glutamate; Cys,
423 cysteine; Orn, ornithine; GSH1, gamma-glutamylcysteine synthetase; GSH, glutathione; Spd,
424 spermidine; GSpdS, glutathionylspermidine synthetase; Gspd, glutathionylspermidine; TryS,
425 trypanothione synthase. **(b)** Growth curves of *L. infantum* WT and $\Delta pnc1$ promastigotes in
426 SDM79 medium supplemented with NA (1, 10 and 100 μ M). Data are representative of three
427 independent experiments. **(c)** Effect of SbIII on the growth kinetics of *L. infantum* WT and
428 $\Delta pnc1$ parasites (supplemented or not with 1, 10 and 100 μ M NA). Data are the mean \pm SD of
429 two biological replicates and are representative of at least five independent experiments. **(d)**
430 Intracellular NAD(H) (left) and NADP(H) (right) levels measured in *L. infantum* WT (black bars)
431 and $\Delta pnc1$ (white bars) parasites after 2 days of culture in the presence of increasing
432 concentrations of NA (quantification of parallel series of cultures as described in Methods
433 section). Values are expressed as Relative Luminescence Units (RLU) for 10⁶ parasites and are
434 the mean \pm SD of triplicate measurements. **(e)** Relative quantification of NAD⁺, NADH, NADP⁺
435 and NADPH in WT and $\Delta pnc1$ parasites after 2 days of growth. Data are the mean \pm SD of
436 duplicate measurements and are representative of two independent experiments. **(f)** Effect
437 of GSH and N-acetyl cysteine (NAC) supplementation on the growth kinetics of *L. infantum* WT
438 and $\Delta pnc1$ parasites in the presence of increasing concentrations of SbIII. Data are the mean
439 \pm SD of duplicate measurements and are representative of three independent experiments.
440 **(g)** Relative gene expression levels of candidate genes involved in SbIII mode of
441 action/resistance in $\Delta pnc1$ parasites relative to the WT strain (see above for abbreviations).
442 The fold-change cut-offs (0.5 and 1.5) are represented by dashed lines.

443 **Figure 2. SbIII-resistant $\Delta pnc1$ parasites show a single amino acid substitution (H451Y) in**
444 **SCAMK gene. (a)** SbIII susceptibility of *L. infantum* WT-SbR and KO-SbR parasites. Growth in

445 the presence of increasing concentrations of SbIII was monitored at 72h by measuring the
446 culture OD at 600 nm. Data are the mean \pm SD of duplicate measurements and are
447 representative of three independent experiments. **(b)** Relative quantification of transcript
448 abundance in WT-SbR relative to WT parasites (left panel) and in KO-SbR relative to $\Delta pnc1$
449 parasites (right panel). The fold-change cut-offs (0.5 and 1.5) are represented by dashed lines.
450 **(c)** Quantification of NAD⁺, NADH, NADP⁺ and NADPH levels in KO-SbR parasites relative to
451 $\Delta pnc1$ parental line after 2 days of growth. Dotted line, ratio = 1. Data are the mean \pm SD RLU
452 ratio values obtained from two biological replicates, each measured in duplicate. **(d)**
453 Comparison of the *LinJ.33.1810* gene sequences in $\Delta pnc1$ (top) and KO-SbR (bottom) parasites
454 showing the C to T substitution (asterisk) in KO-SbR parasites. **(e)** Schematic representation of
455 the functional domains present in the protein encoded by the *LinJ.33.1810* gene using the
456 ScanProsite tool (<https://prosite.expasy.org/scanprosite/>). Lower panel: alignment of the EF-
457 hand domain 3 from *LinJ.33.1810* orthologues in *L. major* (*LmjF.33.1710*), *L. donovani*
458 (*LdBPK_331810.1*), *L. braziliensis* (*LbrM.33.1980*), *Trypanosoma brucei* (*Tb927.2.1820*) and
459 *Trypanosoma cruzi* (*TcCLB.510257.130*). The black arrow indicates the conserved H residue in
460 position 451. The calcium-binding site of EF hand 3 (EF-loop 3) is underlined. **(f)** Position of
461 the sgRNA sequence and the protospacer adjacent motif (PAM) in the *LmjF.33.1710* sequence
462 used to introduce the H451Y mutation in *L. major* with the CRISPR/Cas9 gene editing system.
463 The partial donor DNA sequence illustrates the presence of the targeted and shield mutations.
464 **(g)** Left: schematic representation of the plasmids used to generate the Lm_ctrl and
465 Lm_H451Y strains. Right: chromatograms corresponding to part of the *LmjF.33.1710* gene
466 sequence (Sanger method). The targeted and shield mutations are indicated by a red and a
467 green asterisk, respectively.

468 **Figure 3. The H451Y mutation generates SbIII tolerant and resistant *Leishmania* parasites.**

469 **(a)** Growth curves of Lm_ctrl and Lm_H451Y parasites in SDM79 medium. Data are the mean
470 \pm SD of two biological replicates and are representative of at least three independent
471 experiments. **(b)** SbIII susceptibility of Lm_ctrl and Lm_H451Y parasites. Growth in the
472 presence of increasing concentrations of SbIII (25, 50, 100, 200, 400, 600 μ g/mL) was
473 monitored at 72h by MTT-based assay. Data are the mean \pm SD of two biological replicates
474 and representative of two independent experiments. **(c)** Same growth curves experiments as
475 in (b) showing that Lm_H451Y parasites exposed to the highest SbIII concentrations restart to

476 growth after 7 days. **(d)** Growth curves of Lm-ctrl (left) and Lm_H451Y (right) parasites seeded
477 at 4.10^6 parasites/mL and exposed to cytotoxic concentrations of SbIII (250, 500 and 1000
478 $\mu\text{g}/\text{mL}$) for eight days. Data are the mean \pm SD of duplicate OD measurements and are
479 representative of three independent experiments. **(e)** Quantification of Lm_ctrl and
480 Lm_H451Y parasites viability using MTT-based assays. Parasites were seeded at 4.10^6
481 parasites/mL and exposed to high concentrations of SbIII (100, 250, 500, 1000 $\mu\text{g}/\text{ml}$) for 3
482 and 5 days. Data are expressed as OD 600 nm values and are the mean \pm SD of two biological
483 replicates. **(f)** Light-microscopy images of Lm_ctrl (top panel) and Lm_H451Y (bottom panel)
484 parasites exposed to 1000 $\mu\text{g}/\text{mL}$ of SbIII for 3 days (EVOS FL inverted microscope and x20
485 magnification). **(g)** Integrity of genomic DNA extracted from 5 mL of parasite cultures seeded
486 at 4.10^6 parasites/mL after 3 days of culture in the presence of different SbIII concentrations
487 (0, 250, 500 and 1000 $\mu\text{g}/\text{mL}$). MW: GeneRuler 1kb DNA ladder. **(h)** Lm_ctrl (left) and
488 Lm_H451Y (right) parasites were incubated with increasing concentrations of SbIII (100 to 800
489 $\mu\text{g}/\text{mL}$) for 48h. Parasites were then centrifuged, washed three times with PBS and grown in
490 drug-free medium for ten days. The experiment timeline is shown at the bottom. Parasite
491 density (OD) was checked daily. Data are the mean \pm SD of two duplicate measurements and
492 are representative of two independent experiments. **(i)** Growth inhibition assay of Lm_H451Y
493 parasites cultured with 1000 $\mu\text{g}/\text{mL}$ SbIII for 1 (P1), 5 (P5), 10 (P10) and 15 (P15) consecutive
494 passages.

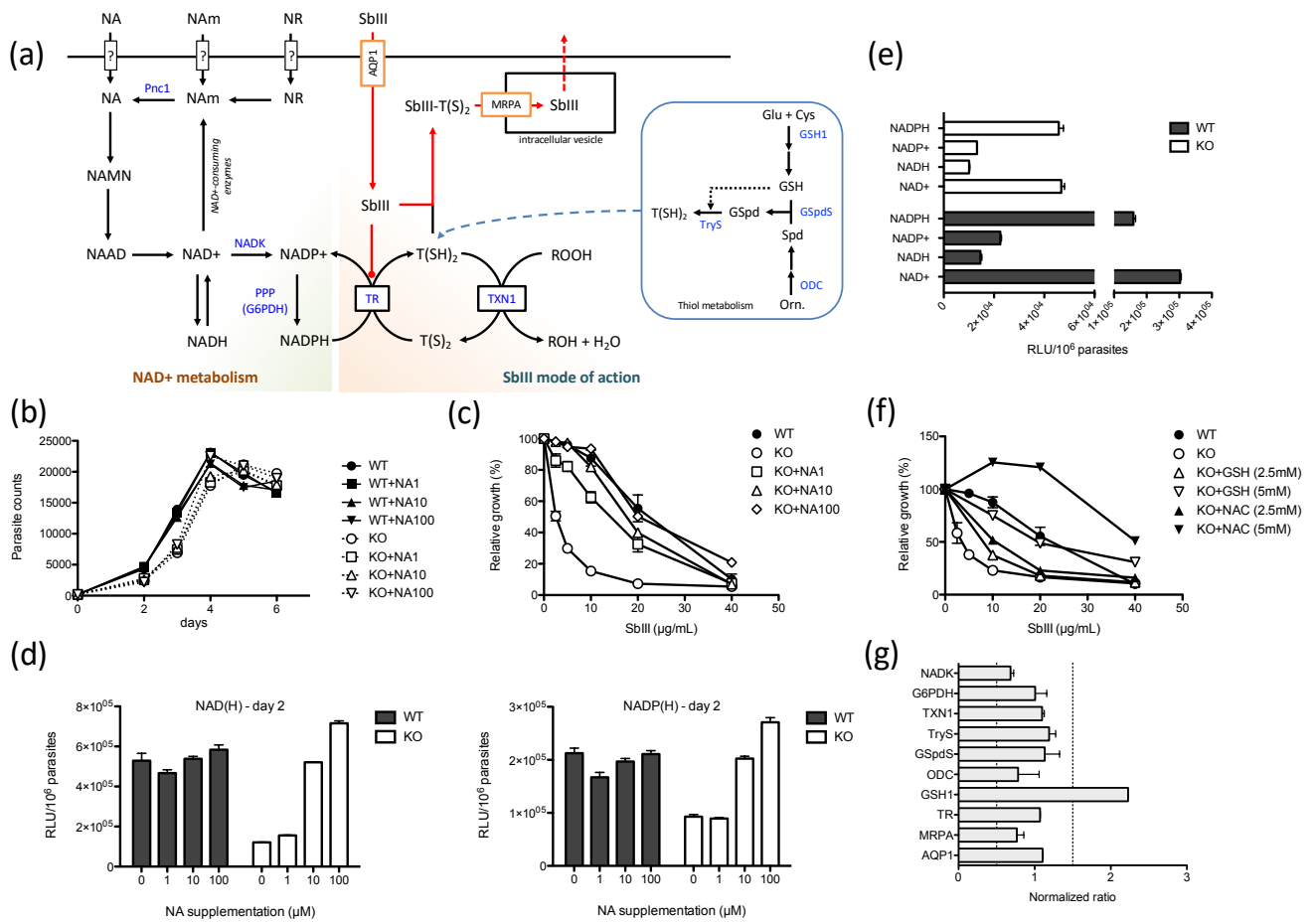


Figure 1

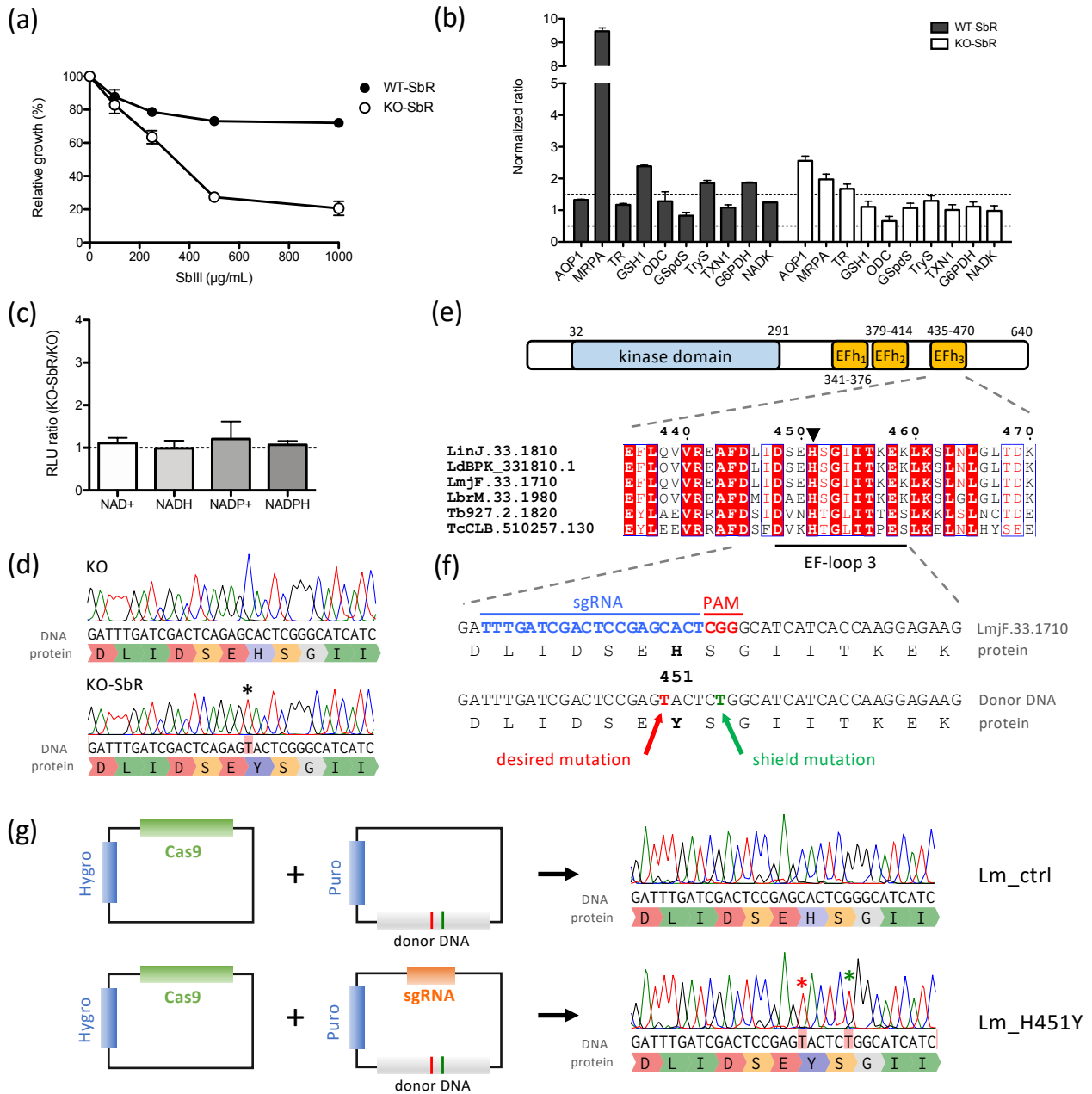


Figure 2

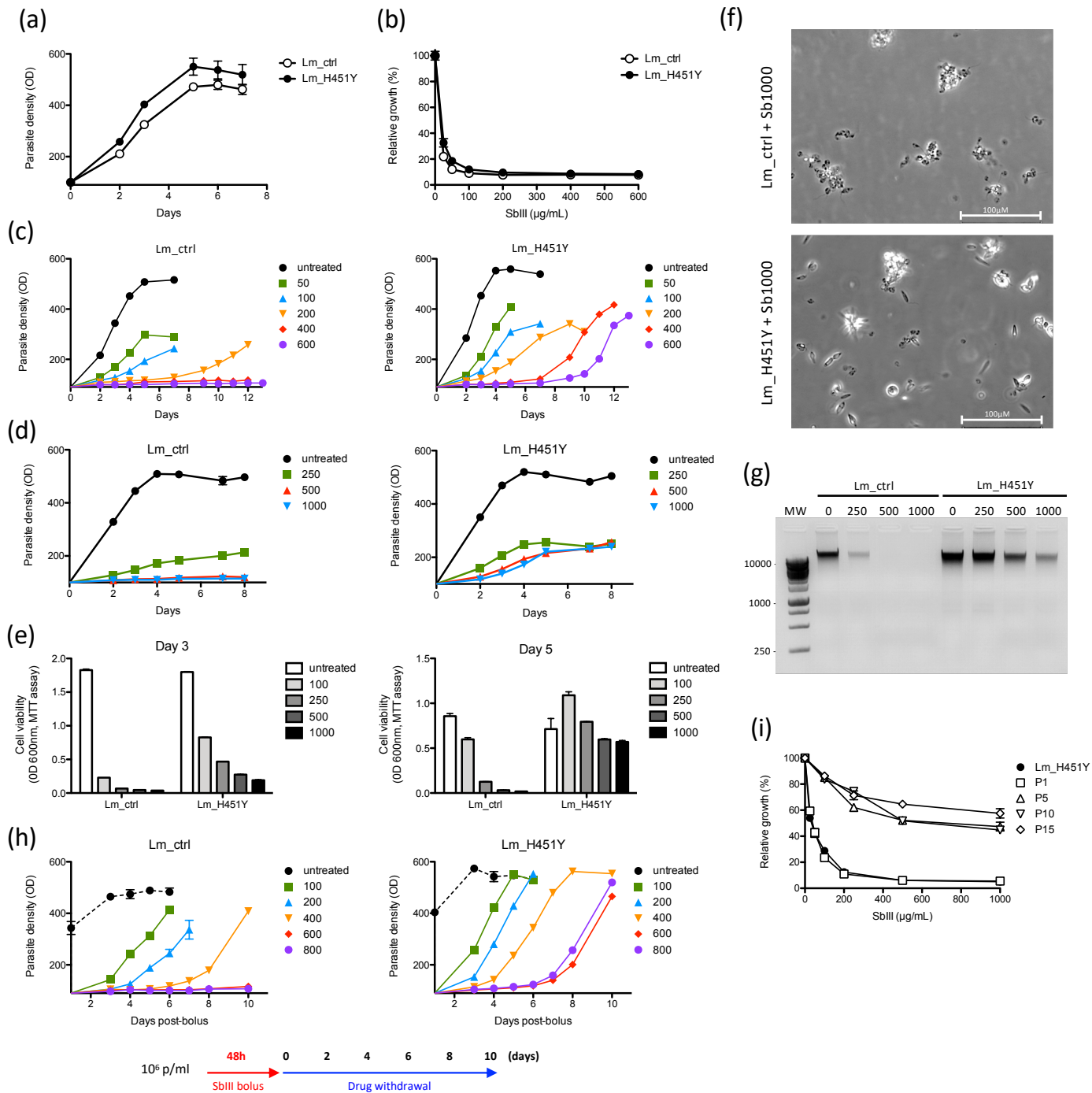


Figure 3

Supplementary data

A single amino acid substitution (H451Y) in *Leishmania* calcium-dependent kinase SCAMK confers high tolerance and resistance to antimony. B Vergnes, E Gazanion, C Mariac, M Du Manoir, L Sollelis, JJ Lopez-Rubio, Y Sterkers, AL Bañuls.

Table S1. Primers used in this study.

Primer name	Sequence (5'-3')
Crispr/Cas9	
Lm_1F	CTGGTACCCATCACCCCTCAGATGAGC (KpnI)
Lm_1R	GATGATGCCAGAGTACTCGG
Lm_2F	CCGAGTACTCTGGCATCATC
Lm_2R	GCTCTAGAGTCGAGAAACCACTTGTGG (XbaI)
seed_F	CAGGCACCGCTGGTGGtttgatcgactccgagcactGTTTTAGAGCTAGAAAT
seed_R	ATTTCTAGCTCTAAAAcagtgtctcggagtcgatcaaaCCACCAGCGGTGCCTG
LmiSCAMK_F	CAACAGCCATCTCACCGTGC
LmiSCAMK_R	GGACAACGGGGTTTCACGTG
qRT-PCR	
gapdh_F	GACACGTCGATCCAGGAGAT
gapdh_R	AGCCCCACTCGTTGTCATAC
GSH1_F	ACTCCCGTACAATGCAGGAC
GSH1_R	GTAGCAGGGGTGAAGAGCTG
ODC_F	CGTCGACCTCTTCTTCCTTG
ODC_R	TCTCGATCGCACTCTTGTTG
TR_F	TGAACAGCATCAACGAGAGC
TR_R	TTGCTCGTGATGCAGAACTC
G6PDH_F	TGCAGATCACGTTCAAGGAG
G6PDH_R	CGGCTCAATGCACTTCAGTA
NADK_F	GATCAGGAAGACCAGGTGGA
NADK_R	ACAACCTTGCTGCAAATCG
GspdS_F	AGTCCGACGCTCTCTGACAT
GspdS_R	GGAGACGTAGTGGTGGGAGA
TRYS_F	AGCCGATGTGGAAAGTCATC
TRYS_R	CCCATAGTTGCCACCAGACT
TXN1_F	TTGAAGCTGCAGAAGCAGAA
TXN1_R	GAAGTCCTCCTCCTCCTCGT
MRPA_F	GGACGTGGAAGAGAGAGTCG
MRPA_R	GTACTIONCGCCATCAGAGAGC
AQP1-F	GAAC TTCACGTCCGAGAACA
AQP1-R	GATGGCCATGTAGCTGGAGT

Table S2. Off-target mutation analysis. List of the off-target candidates identified with the Protospacer Workbench software (with RazerS 3, and five mismatches allowed).

#Target: TTTGATCGACTCCGAGCACT(CGG)
 #Chromosome: LmjF.33
 #Position: 795935
 #Strand: +
 #Doench-Root Activity: 0.177750

target
 NGG PAM
 non-canonical PAM

chromosome	position	strand	sequence	pam	mismatches	dra	score	< 20bp from a specific INDEL in Lm_H451Y
LmjF.33	795935	+	TTTGATCGACTCCGAGCACT	CGG	0	0.17774975685470332	1.0	
LmjF.25	827829	-	CTTGATTGACGCAGAGCACT	TGG	4	0.048381370162212375	0.0022051146716772154	NO
LmjF.31	1158568	-	TTGGATCGACTCAGGGCAGT	AGG	4	0.05825528097973003	0.0005192731021153846	NO
LmjF.30	1324740	-	TGTGAACGACTCCGGGCTCT	GAG	4	0.0	0.000512281696832579	NO
LmjF.36	706422	+	CTTGAGCGACTCAGAGTAGT	AGG	5	0.3237375902104075	0.00028025959499999994	NO
LmjF.16	692860	-	TTTCATCGGCTCCGCGCCA	AAG	5	0.0	0.00012875219199189871	NO
LmjF.04	358636	+	TTTCAGCGACTGAGCAGC	AAG	5	0.0	0.0005212929826708861	NO
LmjF.19	612710	+	TTTCTCGAATCCGACAAC	TAG	5	0.0	0.000565260380487805	NO
LmjF.36	1305474	-	CTTCATCGACTACATGCACT	GGG	5	0.1253521096282401	0.00018433794370370374	NO
LmjF.35	576833	+	TTTGATCGCCTGGGAGCAGG	AGG	5	0.17388963529208032	0.00013826052225180001	NO
LmjF.27	942224	+	ATTGAGCGCCGCGTGCACT	CGG	5	0.15677322326935583	0.0005158850416296295	NO
LmjF.33	796031	+	TTCGATGGACACGGAGCACA	AGG	5	0.04419561195415686	0.0005877012458031232	NO
LmjF.34	950998	+	GTTGCGCGACTCCGAGTGCT	CGG	5	0.2699483343642831	0.00044482702564102556	NO
LmjF.18	722184	-	TTTATTCGACTCAGAGCGCA	CGG	5	0.6556028597619067	0.0003042913397468355	NO
LmjF.30	370497	-	GCAGAACGACTGCGAGCACT	AGG	5	0.21520147439812617	0.002655410685714286	NO
LmjF.36	2457360	+	TTGCAACGACCCGAGCAGC	CAG	5	0.0	0.0013451797386923078	NO
LmjF.36	1570009	-	TTTGGTCTGCTCCGAGCCCG	GGG	5	0.34862116391658615	0.0004744133939999999	NO
LmjF.18	575242	+	TTTCATCCACGCCGTGCACG	TGG	5	0.32245220558738613	0.0005966921620253165	NO
LmjF.27	636627	-	TTTGTTTCGACTCAGGGCGAT	CAG	5	0.0	6.0081526399999974e-05	NO
LmjF.25	411227	-	TATGATCGACTCCGTTTCATG	TAG	5	0.0	5.976304494545457e-05	NO
LmjF.08	529243	+	TTTGATCCTCTCCGAGAGCA	GAG	5	0.0	0.00017604737994216869	NO

Table S3. Heterozygous and homozygous non-synonymous SNPs identified in the coding sequences of KO-SbR mutants but not in the $\Delta pnc1$ parental line.

#CHROM	POS	GENE ID	PRODUCT DESCRIPTION	REF	ALT	AA change	QUAL	GT	DP	RO	AO
LinJ.33	689935	LinJ.33.1810	protein kinase	C	T	H451Y	376,64	1/1	12	0	12
LinJ.29	279621	LinJ.29.0790	heat shock protein 90 (LPG3)	G	A	R592C	293	0/1	18	6	12
LinJ.15	428514	LinJ.15.1070	glutamate dehydrogenase	G	C	Q917E	264,17	0/1	24	12	12

GT=Genotype, DP=Read Depth, RO=Reference allele observation count, AO=Alternate allele observation count

Table S4. Non-synonymous SNPs identified in the coding sequences of Lm_H451Y-SbR mutants, but not in Lm_ctrl and Lm_H451Y parasites.

#CHROM	POS	GENE ID	PRODUCT DESCRIPTION	REF	ALT	AA change	QUAL	GT	DP	RO	AO
LmjF.17	584469	LmjF.17.1200	hypothetical protein	C	T	A75V	61,93	0/1	10	6	4
LmjF.05	19975	LmjF.05.0060	major vault protein	T	A	S647T	61,93	0/1	10	6	4
LmjF.20	352500	LmjF.20.0850	pseudouridine synthase TruD	A	G	E288G	60,29	0/1	10	5	5
LmjF.34	1103815	LmjF.34.2480	hypothetical protein	G	A	stop	55,44	0/1	4	1	3
LmjF.30	915322	LmjF.30.2380	hypothetical protein	A	G	V255A	53,61	0/1	11	7	4

GT=Genotype, DP=Read Depth, RO=Reference allele observation count, AO=Alternate allele observation count

Table S5. Results of the CNV-seq method used to compare CNVs in SbIII-sensitive (KO) and SbIII-resistant (KO-SbR) $\Delta pnc1$ parasites.

CNV	#CHROM	START	END	SIZE (bp)	log2	p.value	COMMENT
CNVR_1	16	751	9750	9000	-1.362885	1.155912e-231	nc tel
CNVR_2	04	116251	122250	6000	0.7291408	8.793945e-86	nc tel
CNVR_3	04	467251	476250	9000	-1.065832	8.215468e-111	nc tel
CNVR_4	11	751	6750	6000	-1.67662	1.353293e-199	nc tel
CNVR_5	17	662251	668250	6000	-1.155018	9.593298e-88	nc tel
CNVR_6	24	863251	869250	6000	-0.8339029	7.110614e-53	nc tel
CNVR_7	32	751	6750	6000	-1.397065	8.888547e-158	nc tel
CNVR_8	35	219751	225750	6000	-0.8113096	4.357229e-54	part of LinJ.35.0530 (ppg5)
CNVR_9	18	716251	722250	6000	-1.402387	1.036969e-108	nc tel
CNVR_10	22	653251	660750	7500	-1.008428	2.584667e-92	nc tel
CNVR_11	02	326251	335250	9000	-0.7970366	1.0524e-81	nc tel
CNVR_12	12	751	8250	7500	-1.738381	0	nc tel
CNVR_13	23	751	6750	6000	-1.995511	0	nc tel
CNVR_14	30	1358251	1367250	9000	-1.276207	4.045936e-143	nc tel
CNVR_15	26	1046251	1052250	6000	-0.8611537	1.727647e-56	nc tel
CNVR_16	13	543751	549750	6000	-1.736291	4.360188e-204	nc inter

nc tel: non-coding telomeric extremity ; nc inter: non-coding intergenic sequence ; ppg5: member of the large and conserved family of proteophosphoglycan.

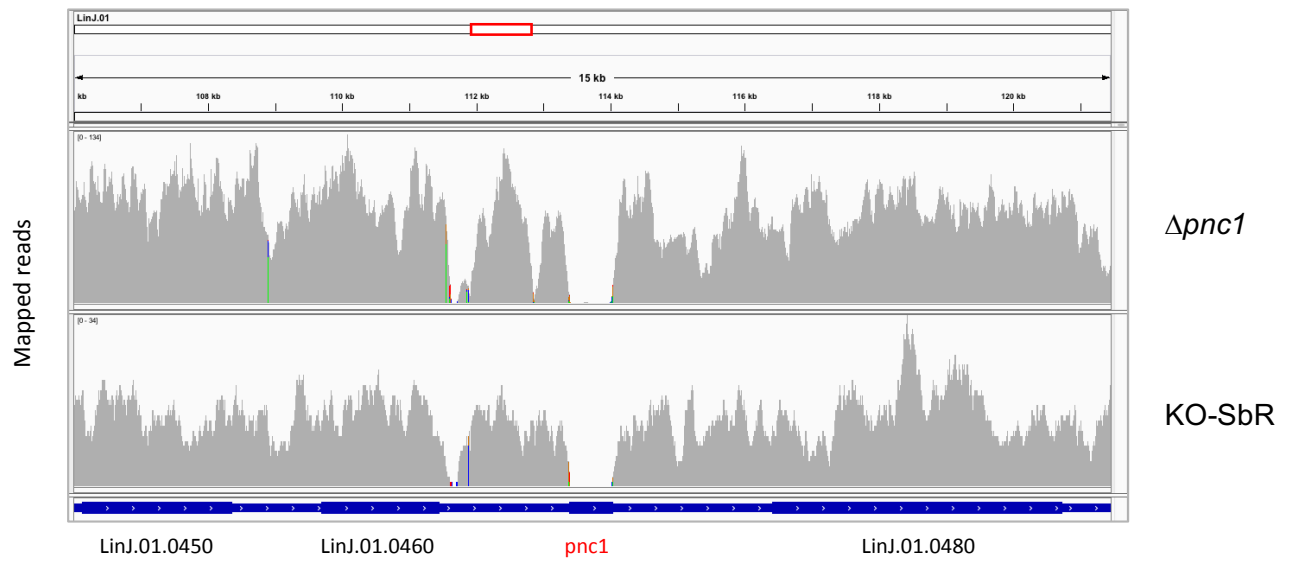


Figure S1. Validation of *pnc1* gene inactivation in sequenced strains. Read coverage (bam files) along chromosome 1 in the $\Delta pnc1$ and KO-SbR strains showing the lack of aligned reads for the *LinJ.01.0470* gene (*pnc1*).

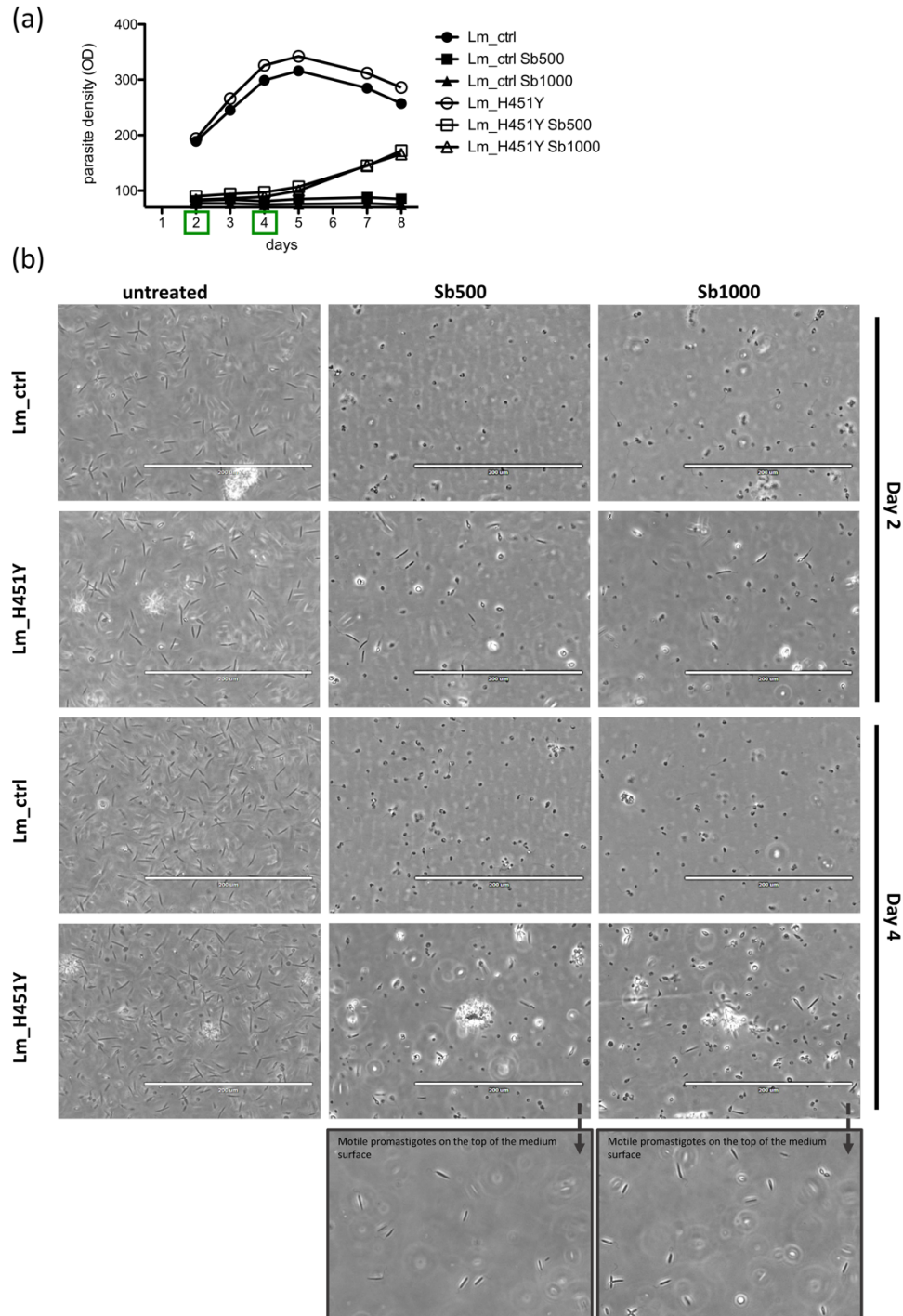


Figure S2. Action of cytotoxic concentrations of SbIII on control and edited parasite populations. (a) Follow-up of parasite density in control (Lm_ctrl) and edited (Lm_H451Y) populations seeded at $4 \cdot 10^6$ parasites/mL and exposed to cytotoxic concentrations of SbIII (500 and 1000 $\mu\text{g/mL}$). (b) Images of the parasite cultures described in (a) taken after 2 and 4 days of growth (EVOS FL inverted microscope with x20 magnification).

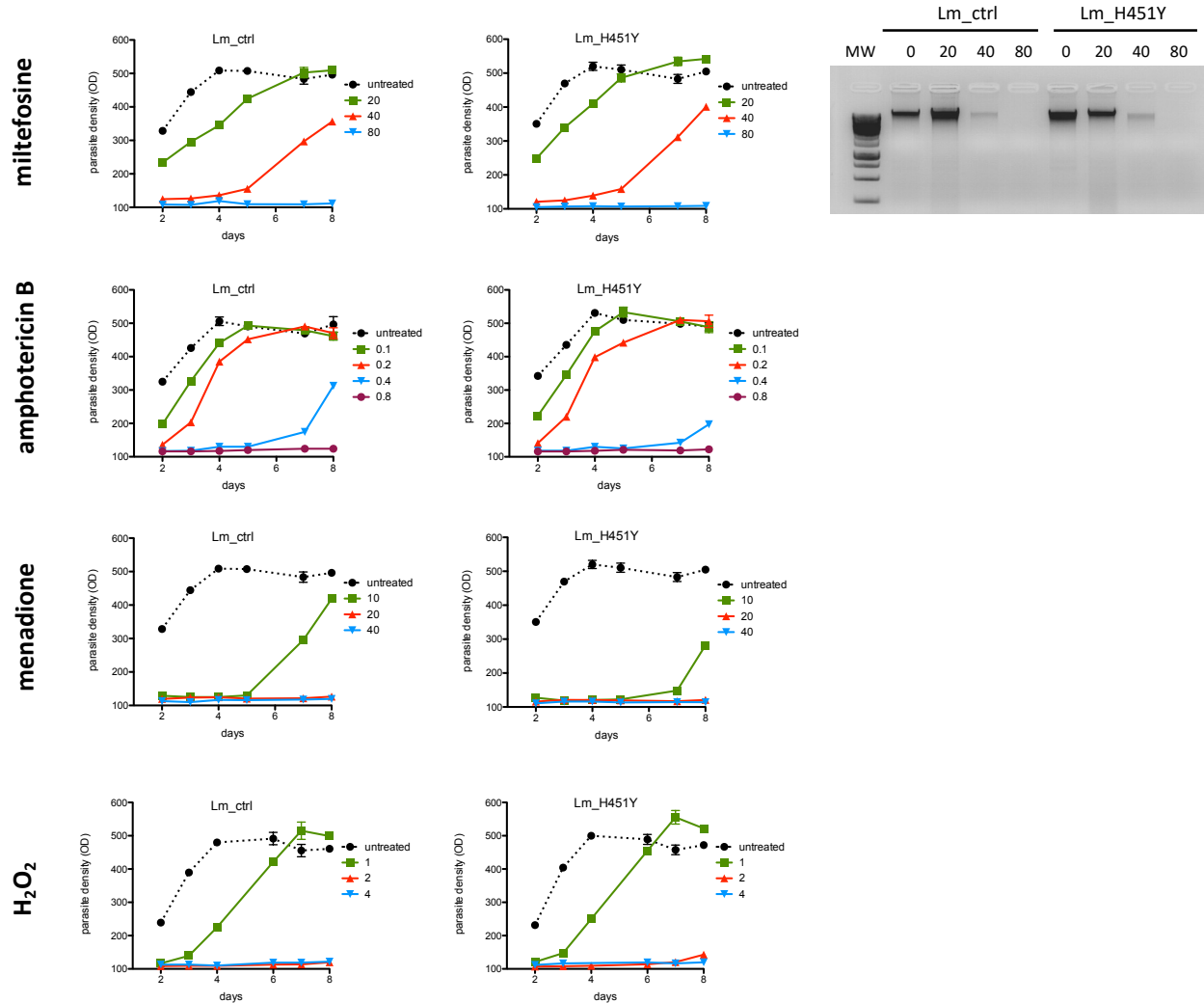


Fig. S3. Growth kinetics of Lm_ctrl and Lm_H451Y parasites exposed to different drugs that induce ROS formation. Similarly to the experiments presented in Fig. 3d, parasites were seeded at $4 \cdot 10^6$ parasites/mL and incubated with cytotoxic concentrations of miltefosine (20, 40, 80 μ M), amphotericin B (0.1, 0.2, 0.4, 0.8 μ M) or menadione (10, 20, 40 μ M). Parasite density was determined daily by OD measurement. For H₂O₂, parasites were incubated with 1, 2, or 4 mM H₂O₂ for 1h, then washed and resuspended in fresh medium. The integrity of genomic DNA extracted from Lm_ctrl and Lm_H451Y cultures exposed to 20, 40 or 80 μ M miltefosine for three days was analysed as described in Fig. 3g.