Multi-drug tolerance in *Leishmania* persister-like cells

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Abstract

Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania* and is found widely across the tropics and sub-tropics, afflicting hundreds of thousands of people. The disease is notoriously difficult to treat. Here, we present evidence of the existence of persister-like cells in cultured *Leishmania* populations, induced upon exposure to normally lethal doses of antimony, a widely used anti-leishmanial. Persisters are a small fraction of non-proliferative cells with reduced metabolism that are adapted to withstand a variety of environmental assaults, including lethal doses of antimicrobials. We show that *Leishmania* persister-like cells survive lethal doses of antimonials by adopting a quiescence phenotype characterised by reduced proliferation, constrained metabolism, and diminished mitochondrial membrane potential. What is more, these cells demonstrate cross-tolerance to other anti-leishmanial drugs. Wild-type persister-like cells reverted to similar levels of drug susceptibility once the antimony-induced pressure was removed. Surprisingly, cells which had previously been selected for genetic changes causing resistance to antimony acquired a level of hyper-resistance after transient passage through the quiescent state, without further genetic change. Our results demonstrate the extreme versatility of this eukaryotic pathogen in adaptation to drug pressure and highlight the need for the development of new anti-leishmanials targeting non-proliferative forms.

Main

The ability of microorganisms to resist the killing action of antimicrobial agents is emerging as one of the most serious public health threats and has been observed for antivirals, antibiotics, antifungals, and also antiparasitic compounds [1]. Understanding the mechanisms that allow them to survive chemotherapy is essential. Several molecular mechanisms can underlie this phenomenon, but the most extensively studied one is drug resistance caused by acquired, heritable genetic changes, usually point mutations or insertion/deletion within genes in which these mutations enable cells to prevent the drug's toxic capacity [2,3]. Importantly, resistant pathogens remain metabolically active and can grow and replicate in the presence of drugs. However, microorganisms can also adopt a physiological state of transient quiescence, manifested by non-proliferation and reduced metabolism. This leads to non-heritable drug tolerance in cases where drug activity depends upon metabolic activities suppressed in quiescence [4]. This can affect whole-microbial populations, but heterogeneity within microbial populations may allow the survival of a small number of specialized quiescent cells, termed 'persisters', in the presence of an antimicrobial agent [5]. After the removal of drug pressure, persisters resume growth and switch back to their drug-sensitive phenotype. The presence of persisters is of particular importance as their quiescent phenotype associated with exposure to a single drug often leads to multidrug tolerance [6–8].

Quiescence is a non-proliferative, physiological resting state that can be adopted by many infectious microorganisms, including bacteria, fungi, and some protozoan parasites as a strategy to survive nutrient limitation, host immunity, and chemotherapy [9]. This reversible state is characterized by an active and regulated cellular and molecular remodelling programme resulting in reduced metabolism, transcription, and translation [9,10]. Importantly, while overall metabolism is switched into an energy-saving mode, crucial processes allowing the survival of the cells under stress conditions remain active, and some processes are even selectively upregulated in the quiescent state [11].

Leishmania are digenetic protozoan parasites that cycle between the sand fly vector and mammalian host. They cause a spectrum of diseases, the most severe form being visceral leishmaniasis (VL), which is lethal if left untreated. *Leishmania donovani* is the etiological agent of VL in the Indian subcontinent (ISC) and in East Africa. Antimonials (Sb) were the first drugs used in the treatment of VL, but they have been replaced first by miltefosine and later by Amphotericin B (AmB) in the ISC due to loss of efficacy attributed to the emergence of drug resistance [12–15]. Phylogenomic analysis of 204 *L. donovani* isolates, followed by experimental studies allowed the identification and characterization of different genomic pre-adaptations to antimonials in *Leishmania* in the ISC [16]. They identified i) a small group of ISC1 parasites, which diverged early and have no obvious genomic pre-adaptation to antimony, ii) a dominant, so-called core group of parasites consisting of several closely related lineages which are pre-adapted to antimony through either the presence of the intra-chromosomal amplification of the H-locus, which contains the MRPA gene, a known driver of antimony resistance or iii) another group with increased H-locus copy number, plus a 2-nt insertion in the AQP1 gene (ISC5), that encodes a key importer of antimony whose loss also contributes to resistance to the drug [16,17].

In terms of drug responses in *Leishmania,* most research has focused on identification of potential drug resistance signatures in natural populations and in experimental evolution studies [16,18–21]. However, recent evidence points to the existence of quiescence and its role in adaptation to drug pressure [22–26]. Quiescence in *Leishmania* was inferred in mice when amastigotes of *L. mexicana* were shown to have a very low replication rate, together with low rates of RNA synthesis, protein turnover,

and membrane lipid synthesis [27]. Another mouse study revealed heterogeneity among *L. major* amastigotes with non-replicating parasites distinguished from slowly replicating populations [28]. Another study of *L. mexicana* in mice revealed quiescent populations within collagen-rich mesothelium surrounding lesions, where miltefosine accumulation was also limited [25]. Using an in vitro antimony-exposure model, we showed that *L. lainsoni* parasites exhibit hallmarks of a quiescence state and that drug-tolerant and persister-like cells of genetically diverse strains of *L. braziliensis* could be detected under high doses of potassium antimonium tartrate (PAT) [23,24]. Recently, Dirkx and colleagues showed that long-term hematopoietic stem cells provide a parasite niche during treatment failure in an animal model of VL infection [29]. Bone marrow stem cell-associated parasites were proposed to enter into quiescence after 2-3 replication cycles with parasites emerging post-quiescence with increased apparent infectivity [26].

In selecting drug resistance in laboratory studies in *Leishmania*, it has become clear that these organisms have particularly plastic genomes and hundreds of karyotypes can emerge from a single clonal population grown in culture [17,21,30–32]. This phenomenon, termed mosaic aneuploidy, is postulated to underlie phenotypic heterogeneity and facilitate adaptation to changing environmental conditions, including drug pressure [32,33]. Generally, persisters in bacteria do not emerge due to genetic changes, however, it is important to ascertain whether a similar process relates to quiescence in *Leishmania* in the selection of drug-tolerant persister-like cells.

In this study, we addressed: i) the role of quiescence and population heterogeneity in the survival of *L. donovani* promastigotes under normally lethal doses of PAT, and ii) whether the presence of genomically defined pre-adaptations to antimony has an impact on the quiescence and persister phenotypes under PAT exposure. We found compelling evidence that quiescence is a universal survival mechanism adapted by genomically distinct *L. donovani* strains. However, the impact of the transition through quiescence, on drug susceptibility differed, depending on the presence of genomic pre-adaptations to antimony. In non-preadapted parasites, the antimony tolerance phenotype was reversible in non-drug conditions. In contrast, in resistant parasites a small sub-population of persisters gave rise to a population of parasites with further increased resistance to antimony. The quiescent state observed in both types of persister-like cells can provide tolerance to PAT, but also other antileishmanial compounds. With this study, we demonstrate for the first time multidrug tolerance in persister-like cells of a eukaryotic pathogen.

Results

Isogenic *L. donovani* lines exposed to normally lethal doses of PAT exhibit intrinsic population heterogeneity in their drug susceptibility

To address whether heterogeneity within populations and quiescence plays a role in response to drug pressure in *L. donovani*, we developed an experimental model, in which we focused on the detection of a small subpopulation of surviving persister-like cells. Specifically, we aimed to identify cells that i) constituted a small subpopulation within isogenic lines, ii) could survive in the presence of a normally lethal dose of a drug and iii) showed a reduction in growth and metabolism associated with quiescent state when compared with the majority of cells. We chose trivalent antimony (PAT), as we showed previously that quiescent cells could be observed in the presence of high doses of this drug in a dermotropic species of *Leishmania*, *L. braziliensis* [23].

We selected ten cloned strains of *L. donovani* isolated in the ISC, with different levels of genomic pre-adaptation to antimony: i) three strains where no obvious genomic changes indicative of pre-adaptation to PAT resistance are known to be present (ISC1); these strains are highly susceptible to PAT; ii) seven strains belonging to different genetic groups (ISC4, 6, and 9) characterized by possessing one known genomic pre-adaptation to PAT, the intra-chromosomal amplification of the H-locus, which contains the MRPA gene, a known driver of antimony resistance; these strains show decreased susceptibility to PAT; iii) one strain with two known genomic pre-adaptations to PAT: a 2-nt insertion in the AQP1 locus, proposed to be associated with antimonial resistance observed in the ISC5 genotype in addition to the intra-chromosomal amplification of the H-locus; the strain shows a low susceptibility to PAT [16,17]. We also included a reference *L. donovani* strain isolated in East Africa (LdBob), highly susceptible to antimony (Table 1) [34,35]. Of note, one of the strains belonging to ISC6 group, BPK085 also carries a different 2-nt insertion mutation in the AQP1 locus, which results in a frameshift [16].

Table 1. Cloned strains of *L. donovani* isolates from the Indian Subcontinent and reference strain (LdBOB) used in this study.

Strain	ISC population	H-locus amplified	AQP1 indel†
Profile 1*			
BPK026	ISC1	no	no
BPK031	ISC1	no	no
BPK156	ISC1	no	no
Profile 2*			
BPK190	ISC4	yes	no
BPK087	ISC4	yes	no
LdBOB	Other	no	no
Profile 3*			
BPK085	ISC6	yes	no
BPK282	ISC6	yes	no
BPK294	ISC9	yes	no
Profile 4*			
BPK080	ISC4	yes	no
BPK275	ISC5	yes	yes

* Profiles 1-4 correspond to patterns of survival under normally lethal PAT dose.
† Indel associated with antimonial resistance.

By using this collection of natural parasite isolates with different levels of genetic pre-adaption to antimony we aimed to study the potential interplay between different heritable, genetically defined forms of drug resistance and quiescence in parasite survival under PAT pressure. We generated rEGFP lines for each of these strains to monitor transcriptional activity of rDNA locus as a negative biomarker of quiescence during exposure to PAT [36].

In the first step, we exposed promastigotes of all lines to a range of PAT concentrations to determine for each strain, the lowest concentration (Supp. Fig.1; Supp. Table 1) at which no metabolic activity could be detected in a resazurin based assay measured at the level of the whole population [37]. By identifying normally lethal doses of PAT specific for each strain, we aimed to establish comparable experimental conditions for each strain in which physiologically quiescent-like cells would be observed, as shown for bacteria. We then subjected each line to the respective strain-specific normally lethal PAT doses over 10 days (with PAT application repeated at day 5), and we monitored viability and growth (Supp. Table 2; Supp. Fig. 2) and established the time-kill curves (Fig.1; Supp. Fig.3) during the exposure. Overall, we identified four broad viability profiles in response to PAT pressure (Fig.1; Supp. Table 2).

Profile 1 (P1) observed in the ISC1 parasites (BPK026, BPK031, BPK156) was characterized by continuous reduction of viability throughout the whole period of exposure to PAT, with the majority of the parasites killed in the first 6 days, while a small remaining population (in the range of 0.7-3.4%) was killed at a slower rate over the last 4 days, allowing those cells to persist until the end of exposure. In profile 2, P2, (BPK190, BPK087, and LdBob) we observed rapid killing of almost the entire population of cells in the first 2 days (1.8-11.0% survived beyond day 2), followed by a slower killing over the following days, with no viable parasites found by day 8. Profile 3 (P3) exhibited by BPK085, BPK282, and BPK294 could be distinguished by fast reduction in viability over the first 3-4 days reaching viability 1.9-3.4%, followed by a slower killing rate until days 6-8. Surprisingly, in the last 2 days of exposure to PAT, a clear increase in the number of viable parasites was observed reaching 5.5-14.9% at day 10. Finally, the last profile (profile 4, P4) displayed by BPK080 and BPK275 is defined by mild reduction in viability, followed by a short period of no visible growth, then a subsequent increase in the number of viable cells. BPK275, a strain with the two genomic pre-adaptations to antimony was the least affected by PAT having a viability of 70% at its lowest value (Supp. Table 2).

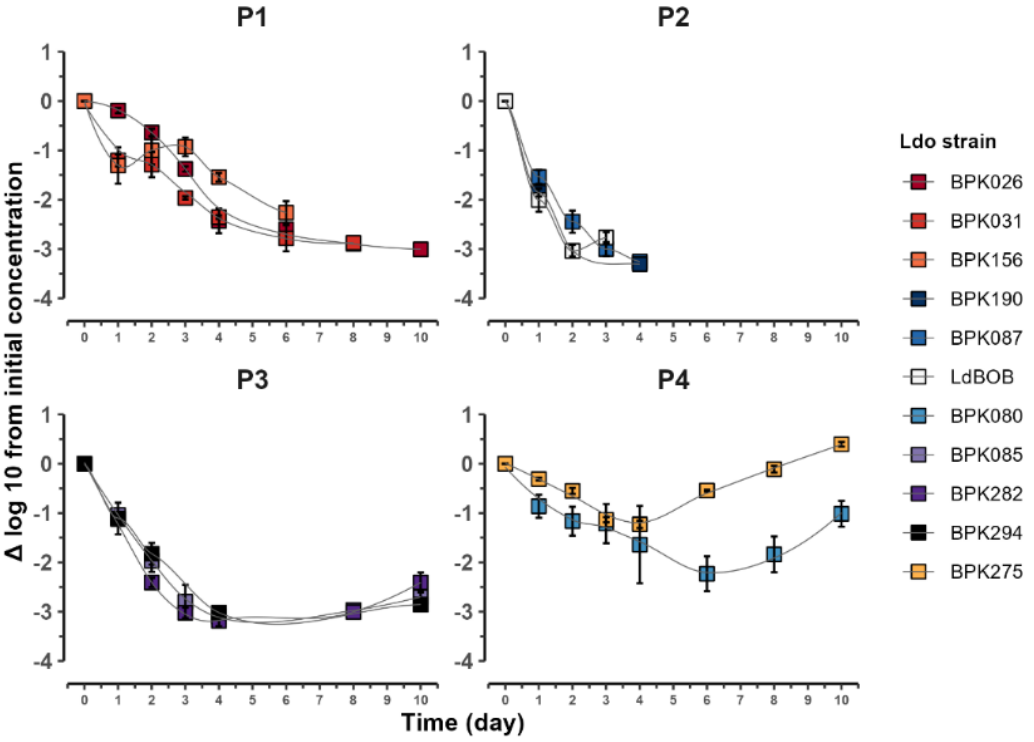

Figure 1. Patterns of survival of *L. donovani* strains under normally lethal PAT concentrations. Time-kill curves under prolonged PAT exposure (see separate strain-specific curves in Supp. Fig.2) showed four different viability profiles (Profile 1-4). Data represents the reduction in promastigote concentration relative to the initial inoculum, the viable parasites were detected by flow cytometry. The results reflect the mean ± SEM of three biological replicates.

***L. donovani* lines adopt a quiescent state to survive PAT pressure**

Given the apparent reduction in growth in all tested lines, we addressed whether the PAT-exposed cells adopt a quiescent state as a survival mechanism to prolonged drug exposure. We addressed this first by analyzing the reduction of rEGFP expression -a proxy of metabolic activity- [36] in all the lines exposed to PAT at day 2, and 4 (PAT D2 and PAT D4, respectively), as compared to proliferative promastigotes. We included, as a positive control, stationary promastigotes (No PAT D4), which are known to have properties of quiescent cells [24]. In all lines of P1/P4 we observed a progressive reduction of rEGFP under PAT pressure, reaching comparable or slightly lower levels to those observed in No PAT D4 (Fig. 2A). In parasites belonging to P2, and P3 measurements were possible only at D2 (all lines in P3 and only BPK087 in P2) due to rapid killing in the lines of these profiles, which resulted in an insufficient number of viable cells for reliable quantification of rEGFP expression. As in other lines, we detected reduction in rEGFP expression at D2 in all three P3 lines. Surprisingly, we observed an upregulation of rEGFP in BPK087 suggesting an inability to enter quiescence under PAT pressure (62.7% of increase, Fig.2A). As expected, all lines that were able to survive drug pressure, resumed normal growth after transfer to standard growth conditions without PAT (Supp. Table 3).

To confirm the quiescent status of the viable cells under PAT, we selected two lines, BPK026 and BPK275, which present the most extreme viability profiles (P1, and P4, respectively) and extreme genomic pre-adaptations to PAT (no known genomic pre-adaptations, and H-locus amplification together with the AQP1 indel associated with antimony resistance, respectively) for more in-depth

193 characterization. First, we measured daily rEGFP expression until day 7, and at the last day of PAT
194 exposure. Both lines showed an overall rEGFP decrease among viable cells over the first 4 days of PAT
195 pressure (Fig. 2B), matching the observed growth arrest (Supp. Fig. 4A). This decrease continued for
196 BPK026 over the next 6 days, reaching a value of 83.1% of reduction in expression. In contrast, rEGFP
197 expression for BPK275 remained stable until day 5, followed by a clear increase on day 10 (Fig. 2B). This
198 is consistent with the apparent reactivation and increase in the percentage of viable and proliferative
199 cells towards the end of drug exposure (Supp. Fig. 4B). Second, we assessed whether parasite
200 replication is arrested/reduced using CellTracker (tracking cell proliferation) combined with SYTOX Blue
201 dye (viability, Fig. 2C). Each round of replication dilutes the CellTracker dye resulting in decreased
202 fluorescence signal, thus fluorescence levels are expected to remain unchanged in non-proliferating
203 cells. In this assay, logarithmic promastigotes were labelled by CellTracker (D0) and either exposed to
204 PAT at a normally lethal dose (PAT) or grown in the control medium (No PAT), and the fluorescence
205 signal was evaluated during short- (D4) and long-term (D10) PAT exposure. In both lines, No PAT
206 parasites showed clear proliferation as indicated by a decrease in fluorescence, reaching 1.97 - 1.57 to
207 1.59 - 1.12 $\log_{10}$ values for BPK026 and BPK275, respectively (Fig. 2C). PAT-exposed parasites by contrast
208 showed less dilution (Fig. 2C). In BPK026, the CellTracker signal decreased slightly from 4.61 to 3.82
209 $\log_{10}$ values at PAT D4, and remained almost the same at PAT D10 (3.61 $\log_{10}$ values) indicating an overall
210 low/no proliferation of PAT-exposed cells. In the case of BPK275, the signal in PAT D4 (2.48 $\log_{10}$ values)
211 decreased as compared to D0 (4.31 $\log_{10}$ values), but it was still higher than fluorescence in the No PAT
212 D4 parasites (1.59 $\log_{10}$ values). No CellTracker signal was detected in PAT D10; this indicates that during
213 the early exposure to PAT, BPK275 parasites transiently reduce their proliferation, while resuming active
214 proliferation later.

215 Next, we investigated the mitochondrial membrane potential as an indicator of mitochondrial
216 activity, which is known to be reduced in quiescent *Leishmania* cells [24]. We labelled promastigotes at
217 different time points under PAT exposure and their respective controls, with MitoProbe™ DilC, a
218 permeable cationic dye that accumulates across the mitochondrial membrane when the interior of the
219 mitochondrion is negatively charged compared to its outside (Fig. 2D). In the absence of PAT, BPK026
220 showed high potential in logarithmic promastigotes, and this decreased significantly in the stationary
221 phase. Unexpectedly, in BPK275, the mitochondrial membrane potential was much lower in logarithmic
222 parasites in comparison to BPK026 (29,569 ± 2,071 and 10,142 ± 2147 MFI values, in BPK026 and
223 BPK275, respectively, Fig. 2D). Under PAT pressure, patterns were also markedly different between
224 lines. In BPK026, early PAT-exposed promastigotes showed a similar potential to late stationary (D10)
225 promastigotes, which further decreased to 899 ± 165 MFI values at day 10 (Fig. 2D). In the PAT-exposed
226 BPK275, the mitochondrial potential was the lowest at D2 (4,326 ± 47 MFI value), and it gradually
227 increased reaching 12,611 ± 1,741 MFI value at D10 (Fig. 2D).

228 Thus, based on the rEGFP, CellTracker, and mitochondrial potential measurements, we concluded
229 that both lines exhibited features of quiescence during their exposure to normally lethal doses of PAT.
230 Though in BPK275, the line with genetic resistance to PAT, the period of quiescence was transient, and
231 the quiescence state appeared to be less deep. The accumulated data demonstrates that although
232 different dynamics of killing could be observed in *L. donovani* promastigotes exposed to lethal doses of
233 PAT, in all lines that survived PAT pressure, adoption of a quiescent state was associated with survival.

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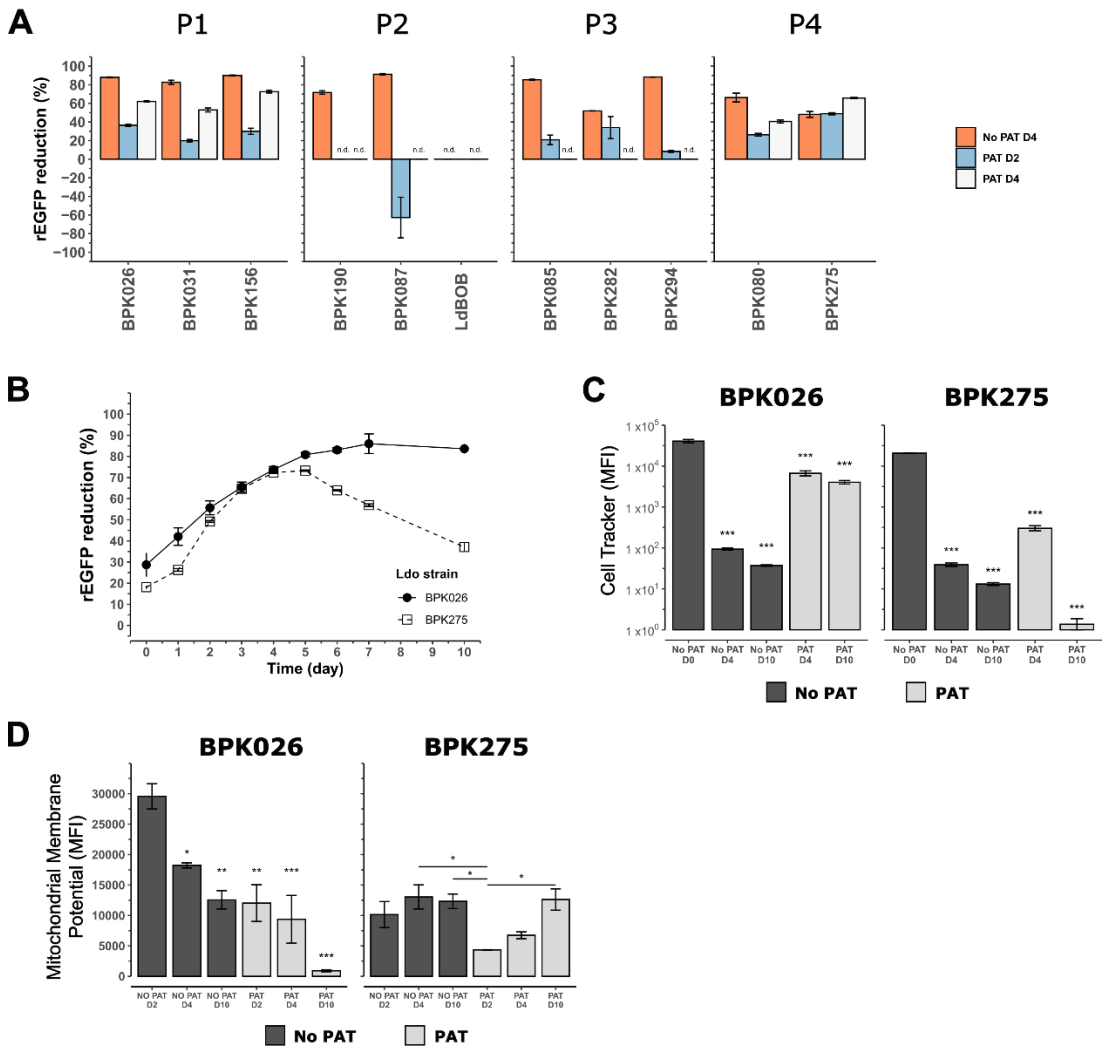

Figure 2. Quiescence features of *L. donovani* promastigotes under prolonged PAT exposure. (**A**) rEGFP expression reduction in percentage in P1-P4 profiles under normally lethal doses of PAT exposure for 2 (PAT D2) and 4 (PAT D4) days. A group without exposure for 4 days was included (No PAT D4). In certain samples, rEGFP expression data is not reported due to a limited number of viable cells, indicated as 'n.d.' (not determined). (**B**) rEGFP expression reduction in percentage in BPK026 and BPK25 from day 0 to 10. (**C**) Proliferation status measured by CellTracker in promastigotes from BPK026 and BPK275 under PAT exposure (PAT) with the respective no-drug controls (No PAT). The results were compared to the initial staining point of the parasites (No PAT D0). (**D**) Measurement of the mitochondrial membrane potential with MitoProbe ™ DilC by flow cytometry in promastigotes exposed for 2, 4, and 10 days to strain-specific PAT normally lethal dose (PAT); and the respective controls without drug (No PAT). The results were compared to promastigotes at the logarithmic state (No PAT D2). (**A,B**) The rEGFP expression reduction in percentage was calculated by comparison of the signal of each group to promastigotes without PAT exposure at the logarithmic state. The data reflects the mean ± SEM of three biological replicates. The asterisks represent the statistically significant difference after Tukey's post-hoc test; * P < 0.05, ** P < 0.01, *** P < 0.001.

<u>The impact of transition through quiescence during exposure to PAT on antimony susceptibility depends on the presence of genomic pre-adaptations to antimony in analysed *L. donovani* lines</u>

Although the quiescent state was found in all but one of the studied lines, the different killing profiles observed earlier, suggested different survival mechanisms. Specifically, P1 parasites seemed to behave similarly to classical bacterial persisters with no growth during exposure to the drug, while P3 and P4 parasites were able to resume growth under drug exposure after a short transition through the quiescent state, suggesting that a small subpopulation of these resistant parasites became hyper-resistant. To ascertain whether drug-pressure was selecting for altered genome sequences of parasites

257 isolated post-exposure to PAT, we analysed the PAT-recovered parasites with particular focus on their
258 PAT susceptibility and potential genomic alterations.

259 First, we measured the PAT susceptibility of the cells that recovered from the PAT exposure by
260 comparing the IC_{50} without drug exposure (No PAT) and after removal of PAT and recovery of growth
261 (Post-PAT) (Table 2). As reported previously, we found that the P1 parasites (ISC1 group) showed the
262 highest susceptibility, with IC_{50} values ranging between 9 ± 0.6 and 19 ± 1.2µM. The P3 and P4 parasites
263 exhibited a lower susceptibility, with IC_{50} values ranging between 46.4 ± 0.5 µM and 137.1 ± 11.9 µM.
264 When control and Post-PAT conditions were compared, two patterns were observed: (i) P1 lines did not
265 show changes in IC_{50} after the exposure to PAT; (ii) in contrast, the P3 and P4 lines showed a significant
266 increase of IC_{50} in Post-PAT conditions. The result observed for the P1 parasites is consistent with the
267 classical behaviour reported for persister cells in bacteria. However, the extreme increase in IC_{50} values
268 in such a short period of time observed for P3/P4 parasites was unexpected, but consistent with the
269 ability of these parasites to grow in the presence of PAT.

270

271 **Table 2.** Drug susceptibility of *L. donovani* promastigotes (IC50, µM) of ten ISC and one reference strain measured by resazurin
272 assay after removing normally lethal dose 10-day of PAT (post-PAT); the control (No PAT) was maintained in parallel and not
273 exposed to the drug. For three lines that did not recover after drug exposure, the data is not reported and denoted as 'n.d.'
274 (not determined). The data is presented as the mean ± SEM of three biological and technical replicates.

Strain	H-locus amplified	AQP1 indel†	IC50 PAT No PAT	IC50 PAT Post-PAT
P1				
BPK026	no	no	9.0 ± 0.6	6.7 ± 0.7
BPK031	no	no	19.0 ± 1.2	20.9 ± 4.6
BPK156	no	no	18.2 ± 0.7	19.7 ± 0.1
P2				
BPK190	yes	no	nd	nd
BPK087	yes	no	nd	nd
LdBOB	no	no	nd	nd
P3				
BPK085	yes	no	89.9 ± 0.5	241.5 ± 14.7
BPK282	yes	no	137.1 ± 11.9	423.5 ± 2.8
BPK294	yes	no	40.2 ± 4.4	230.1 ± 13.9
P4				
BPK080	yes	no	52 ± 1.7	86.8 ± 2.6
BPK275	yes	yes	46.4 ± 0.5	214.1 ± 30.1

275 † Indel associated with antimonial resistance.

276

277 We then, we sequenced and compared the genome of control and Post-PAT, in triplicates, in order
278 to search for possible signatures of drug resistance. In our analysis, we focused on potential changes
279 of SNPs, INDELs, copy number of individual genes and the H-locus and somy values. Because of ISC1
280 strains/P1 parasites having much higher number of heterozygous SNPs in comparison to other strains
281 [16], we carried out the analysis of P1 and P3/4 parasites separately. In both P1 and P3/4 lines, we did
282 not observe consistent (*i.e.* present in all three replicates) SNP/indel changes (Fig. 3A,B; Supp. Fig. 5)
283 changes. Similarly, there were no consistent changes in the depth of individual coding sequences (Fig.
284 4A,B; Supp. Fig. 6), including the MRPA genes (Supp. Table 4) when No- and Post-PAT parasites were
285 compared. However, when we analyzed somy changes in Post-PAT parasites as compared to non-
286 exposed lines, we observed a general decrease of somy of several chromosomes in both P1 and P3/4

287 lines (Fig. 5; Supp. Fig. 7). Focusing on the somy changes that we consider biologically significant (S
288 values difference of at least 0.5, p-value≤0.05), we detected: i) decrease in at least one chromosome
289 in all P1 parasites, namely chr 33 and chr07 in BPK026, chr 23 in BPK031 and chr 09 in BPK156. In
290 addition, five chromosomes, chr 02, chr 08, chr 14, chr 32and chr 35 were decreased in BPK085, and
291 chr 26 in BPK294 ii) no significant increases in chromosome copy number were found in P1 parasites,
292 while we observed a significant increase in copy number of chr 6 and ch31 in BPK282 and BPK275. In
293 addition, an increase (albeit not reaching statistical significance) in chr 31 was found in BPK294, where
294 chr 07 was also increased. No significant aneuploidy changes were detected in BPK080.

295 In summary, no consistent genomic alterations could be detected in either P1 or P3/4 parasites.
296 This further supports persister-like nature of a small population in P1 parasites that survive normally
297 lethal doses of PAT. In the case of P3/P4 parasites, we report here detection of a unique form of
298 transient persisters with the ability to retain their reduced PAT susceptibility in drug-free conditions
299 showing no discernible genetic change when compared to the precursors. Further investigation,
300 possibly into epigenetic modification is required to ascertain the mechanism allowing the growth in
301 PAT and increase of IC_{50} in recovered P3/P4 parasites.

302 Overall, the impact of the transition through quiescence, on drug susceptibility differed, depending
303 on the presence of genomic pre-adaptations to antimony. Lines without any genomic pre-adaptation
304 exhibited features of a classical quiescence phenomenon linked to the presence of persisters, where
305 increased tolerance to a drug is restricted to the quiescence phase. Consistent with this notion, no clear
306 drug resistance signatures were detected in Post-PAT parasites. While in the lines that were genomically
307 pre-adapted, after a short period of quiescence, a subpopulation of more tolerant cells was able to
308 resume growth even in the presence of PAT. These emergent cells had a level of PAT resistance beyond
309 the level of the parent clone in which quiescence was initially induced. However, no consistent genomic
310 changes could be detected in the analyzed P3/P4 parasites. This suggests a presence of a unique form
311 of adaptation in these lines, in which the exact molecular mechanism underlying the reduced PAT
312 susceptibility remains to be characterized.

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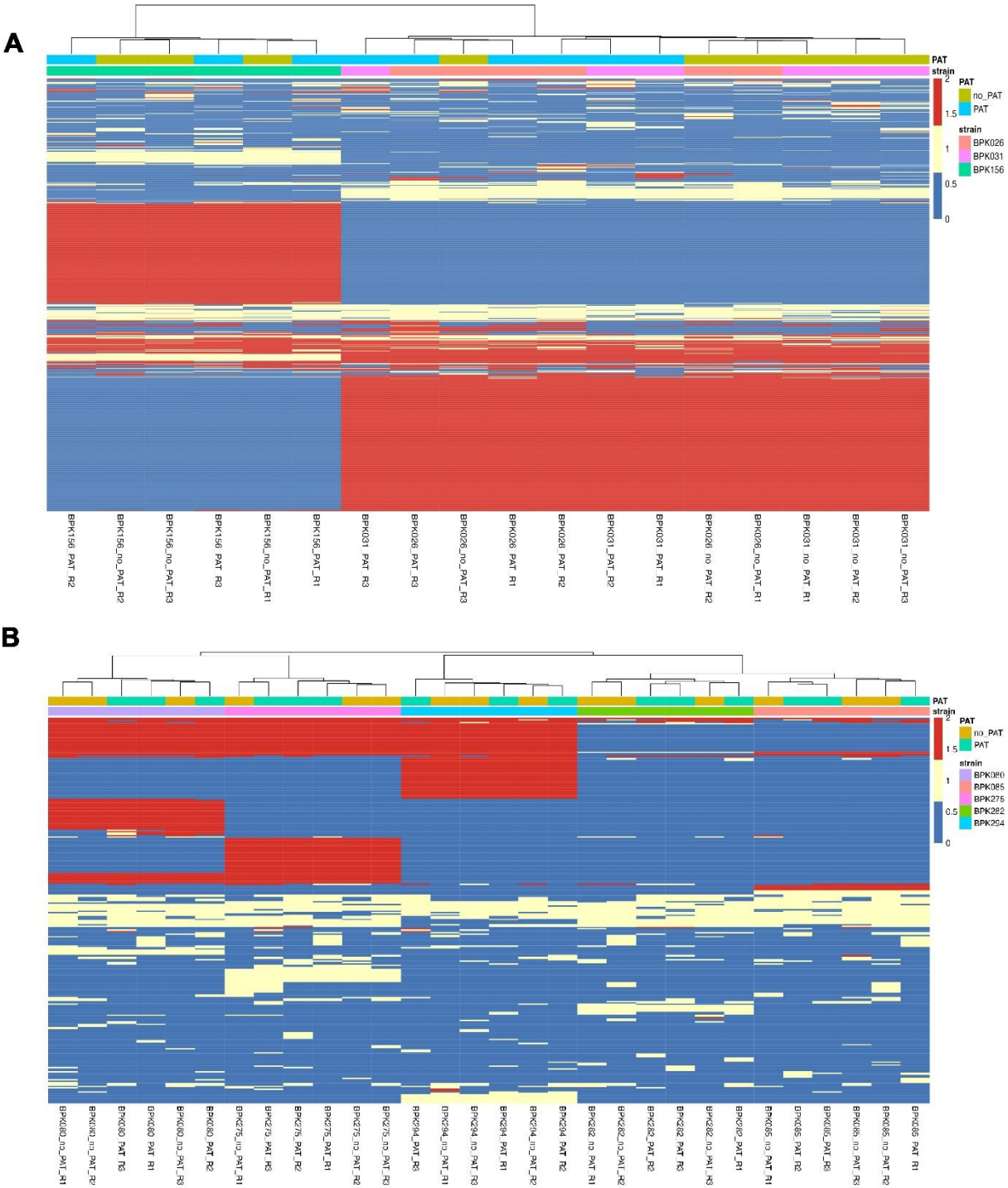

Figure 3. Heatmap illustrating the distribution of single nucleotide polymorphisms (SNPs) across strains belonging to (**A**) Profile 1 and (**B**) Profiles 3/4. SNPs that exhibited no variation across the 30 Profiles 3/4 samples or the 18 Profile 1 samples were filtered out. The color scheme categorizes SNPs as follows: blue represents the absence of a SNPs, yellow indicates heterozygous SNPs, and red denotes homozygous SNPs. Hierarchical clustering of samples (columns) and SNPs (rows) was performed using the complete linkage method with Euclidean distance. A color-coded bar above the heatmap indicates whether each strain was exposed to PAT, alongside the strain names. The data represents three biological replicates (R1, R2, R3) after removing normally lethal doses of PAT (PAT), and their respective control without drug (no_PAT).

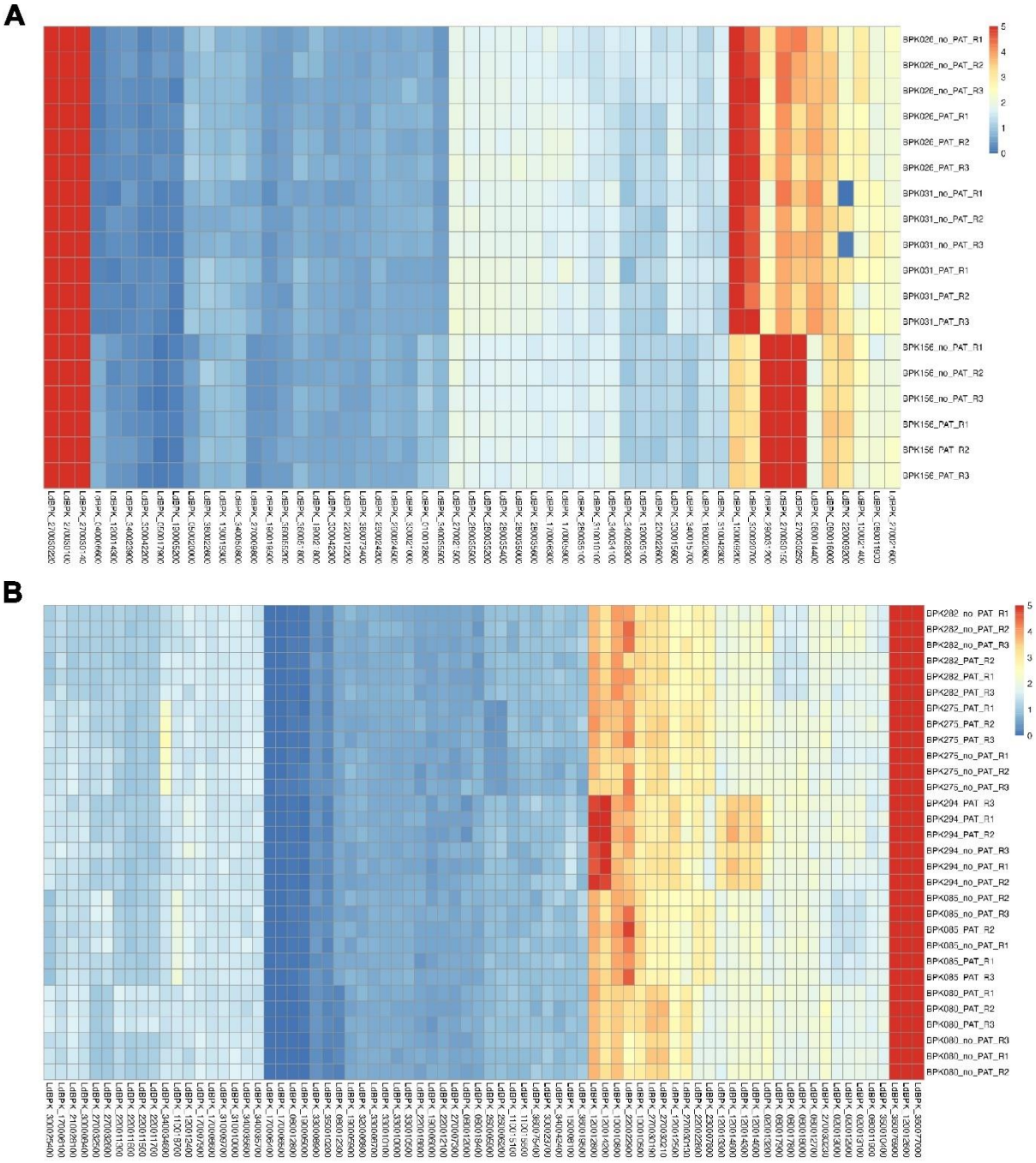

Figure 4. **Heatmap of the** (**A**) Profile 1 and (**B**) Profiles 3/4 strains, displaying CNV values for genes with a statistically significant 50% increase or decrease in CNV. Statistical significance was determined using a Student's t-test to compare CNV values between PAT-exposed (PAT), and non-exposed replicates (No_PAT) for each strain. Three biological replicates were included (R1,R2,R3) for each strain and drug exposure.

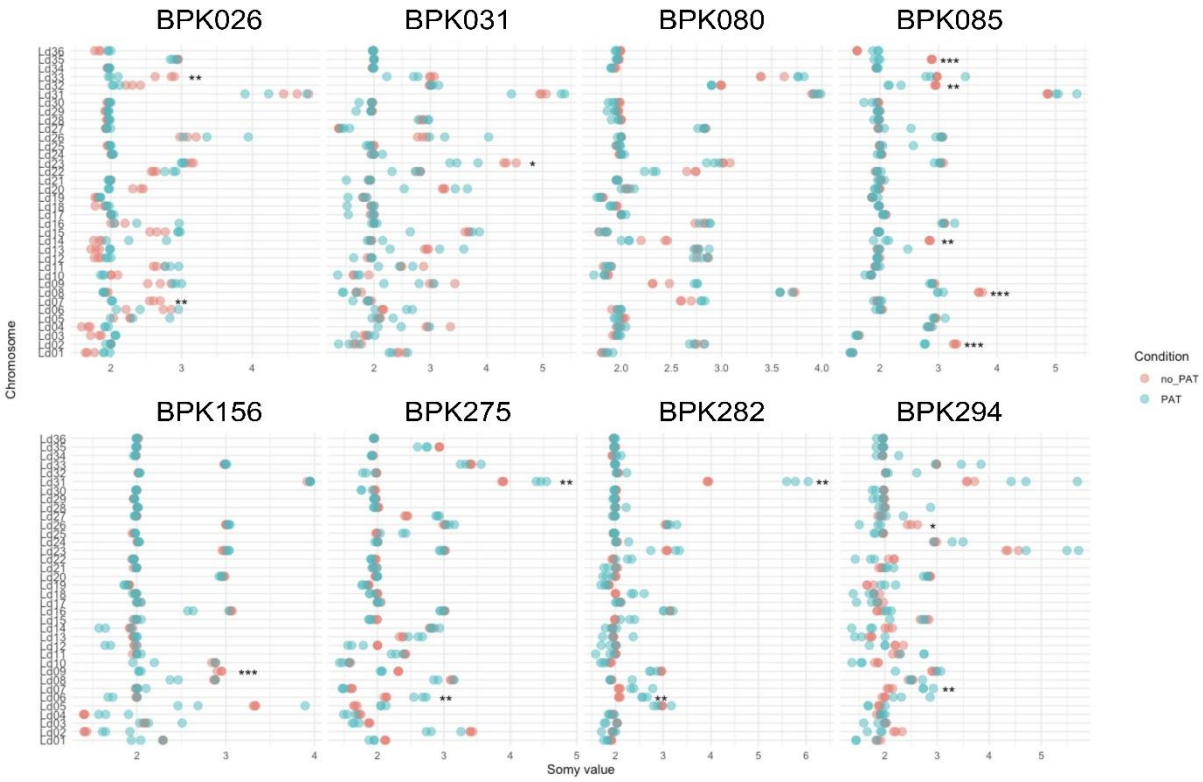

Figure 5. Dot plot containing the somy values per chromosome and per strain. For each chromosome and each strain, the somy value is indicated in the non-exposed samples (red, No_PAT) and the PAT exposed sample (green, PAT). Statistically significant somy differences using student t-test between non-exposed versus PAT-exposed samples are indicated by asterisks per chromosome and strain (* < 0.05, ** < 0.01, *** < 0.001). The data represents the values of three biological replicates.

PAT-tolerant promastigotes show cross-tolerance to other drugs

In bacteria, entering into a non-proliferative physiological state after the exposure to one drug, can provide tolerance to other antibiotics [6–8]. Hence, we tested whether the observed PAT tolerance, associated with the acquired quiescent state in the BPK026 and BPK275 lines, would also provide tolerance to other known antileishmanial compounds. We, therefore, evaluated the susceptibility of PAT-tolerant promastigotes of both BPK026 and BPK275 towards 5 compounds with different modes of action: amphotericin B (AMB), miltefosine (MIL), bortezomib (BOR), carbonycil (FCCP) and paromomycin (PARO). Briefly, i) AMB binds ergosterols and potentially destabilises membranes, ii) MIL is a lipid binding compound and likely also acts through destabilization of membranes, iii) BOR is a proteasome inhibitor, iv) FCCP is a protonophore, which can permeabilise the mitochondrial membrane, and v) PARO is a probable protein synthesis inhibitor [38–42]. First, we found that the IC_{50} values for each of these compounds were comparable in BPK026 and BPK275 (Supp. Table 5). To assess the protective effect of PAT-associated tolerance, we first triggered quiescence by exposing the promastigotes to PAT (strain-specific normally lethal doses) for 5 days, followed by 5 days of simultaneous exposure of PAT and the additional drug (serial exposure (S), Supp. Table 6). Secondly, we added both PAT and the second drug from the very beginning (combination exposure (Co), Supp. Table.6). The drugs were applied at their respective IC_{50} concentrations in both Co and S conditions. As a control exposure, we repeatedly treated with PAT during the first and second exposure (Supp. Table 6). To assess the killing effect of these drugs, we quantified the % of viable promastigotes in relation to the control exposure (C).

353 In the case of BPK026, two main patterns were observed. On one hand, with AMB, MIL, and BOR,
354 there was a strong reduction of the viability ratio in respective combination exposures (0.001 to 0.34
355 range), while in the serial exposures, the viability was comparable to the levels in control treatment
356 (0.79-0.82 values in S, and 1.0 in C; Fig.6A). This indicates that PAT-triggered BPK026 quiescent cells
357 gain tolerance to AMB, MIL, and BOR. On the other hand, with FCCP and PARO, viability remained
358 similar to control exposure in both Co and S conditions, suggesting that used concentrations of FCPP
359 and PARO were too low to enhance PAT killing (Fig. 6A). Results were confirmed by the reversibility
360 assay: *i.e.* the combination of PAT+AMB for which growth was observed in only one out of three
361 replicates, while all three replicates demonstrated proliferation in the case of serial exposure (Table 3).
362 In the case of BPK275, results were different and 5 patterns were observed (Fig. 6A): (i) for BOR, a low
363 viability ratio in Co (0.047 ± 0.02 value), and comparable viability in the C exposure and S exposures,
364 suggesting cross-tolerance to BOR in PAT-exposed quiescent cells, ii) lower viability ratios for AMB (both
365 Co and S exposures) in comparison to C, but the viability in S was significantly higher than in Co,
366 suggesting some level of protection against AMB provided by quiescence of PAT-exposed cells (iii) same
367 viability as control for MIL (both Co and S conditions), indicating that used MIL concentrations were too
368 low to enhance PAT killing, (iv) for FCCP, although low viability in S was observed, microscopic
369 examination revealed an abundance of parasites, suggesting that parasites likely died due to nutrient
370 deprivation, and (v) for PARO a higher viability ratio in Co exposure in comparison to C and a ratio close
371 to the control under serial exposure, suggesting that PARO and PAT act synergistically to trigger the
372 presence of quiescent cells. In all conditions, the reversibility assay revealed proliferation in the 3
373 replicates (Table 3). Collectively, these results show that PAT-tolerant cells also show tolerance to other
374 drugs, but the degree of this cross-tolerance is drug- and strain-dependent.

375 To analyze the cross-tolerance further we focused on protection against BOR, for which we also
376 executed combination and serial exposure and compared the effects of applying BOR at 3 different
377 concentrations: at 1x, 2x, and 5x IC_{50} of BOR (see experimental design in Supp. Table 7). In the case of
378 BPK026, viability ratios slightly decreased with the increasing concentration of BOR in the combination,
379 condition, but this did not affect the observed cross-tolerance in S exposure (Fig. 6B). In contrast, in
380 BPK275, viability ratios decreased with the increase of BOR concentration in both conditions, with
381 almost no viable cells detected in the two highest concentrations of BOR. (Fig. 6B). The reversibility
382 assays showed that none of the Co replicates could grow, while a few replicates in the S condition could
383 re-proliferate in BPK026. By contrast, for BPK275, all replicates in the S condition could reactivate, while
384 only two or none were able to proliferate in the higher concentrations of BOR in Co exposure (Table 3).
385 This indicates that cross-tolerance to BOR could be observed for both strains, but it was more
386 pronounced for the antimony resistant BPK275 line.

387

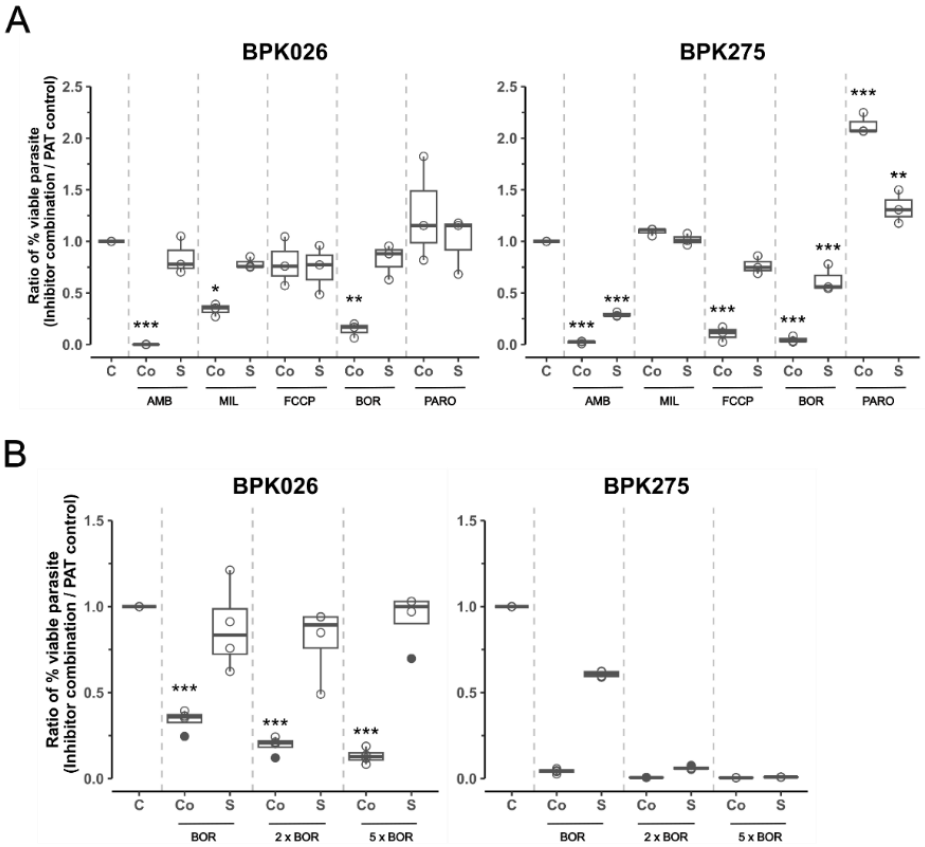

Figure 6. Survival of PAT-tolerant *Leishmania* promastigotes when exposed to other drugs. (**A**) Ratio of percentages of viable parasites submitted to different compounds (IC50) vs normally lethal doses of PAT (control, C) and following two drugging schemes (Co, combination and S, serial); (i) MIL, Miltefosine; AmB, Amphotericin B, PARO, Paromomycin; BOR, Bortezomib and FCCP, Carbonycil. Plotted data shows the mean ± SEM of three biological replicates for each combination or serial. (**B**) Ratio of percentages of viable parasites submitted to BOR (1x IC50, 2x and 5x IC50) vs normally lethal doses of PAT (control, C) and following two drugging schemes (Co and S). Plotted data shows the mean ± SEM of four biological replicates for each combination or serial. The asterisks indicate statistically significant differences for each scheme compared to the control, analyzed by Tukey's post-hoc test (**A**) and Student's t-test, (**B**)* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

This study demonstrates that genetically different *L. donovani* strains use quiescence as a survival strategy under normally lethal doses of trivalent antimony. In most lines, upon exposure to PAT a small non-replicating population with reduced metabolism was observed and had the ability to survive the treatment. A quiescence state was also found in an antimony resistant *Leishmania* line, in which a majority of cells survived drug exposure. Quiescence was detected in all parasites with genomic pre-adaptation to antimony, and these cells could also resume growth while still under treatment exposure, enhancing the level of resistance to PAT in so doing. Quiescence, therefore, appears to be a widespread phenomenon in *Leishmania* parasites exposed to normally lethal doses of a drug.

Here we triggered entry into quiescence by exposure to antimony, analogous to triggered persistence in bacteria rather than spontaneous persistence [4]. All lines surviving normally lethal doses of PAT, showed a bi-phasic time-kill curves typical for bacterial persister cells [43,44], though in two lines, BPK275 and BPK080, the subpopulation of PAT tolerant cells was larger than typical. Detailed analysis of the lines with the most extreme survival phenotypes revealed several properties typical of persisters, including reduction of metabolism, proliferation and mitochondrial membrane potential with no

414 apparent genomic changes and cross-tolerance to other antileishmanial drugs. However, the ability to
415 resume growth after a transient adoption of quiescence observed in lines with pre-existing antimony
416 resistance is inconsistent with classical definition of bacterial persisters, since these lines acquire a
417 further reduction in sensitivity to drug during the passage through quiescence. Because of this unique
418 mixture of observed phenotypes in the quiescent *L. donovani* lines, we collectively call them persister-
419 like cells.

420 Parasites without known antimony resistance, when triggered to enter quiescence over a 10-day
421 period remain quiescent while still under drug treatment, and resume growth only when transferred
422 to antimony-free medium. By contrast, *Leishmania* that had harboured genetic mutations that yielded
423 resistance to antimony, also entered quiescence when exposed to antinomy at a dose beyond that to
424 which they are normally resistant. However, these cells were able to re-enter growth phase whilst in
425 medium containing drug. Remarkably, these parasites that resume growth whilst still in medium
426 containing antimony are actually more resistant to the drug than they were before having passed
427 through quiescence. Although we do not have a molecular explanation for this, it is possible that the
428 MRPA pump which removes antimony from the cells [45], and is amplified as part of the H-locus in
429 resistant cells is expressed at still higher levels independently of gene copy number (which was not
430 further amplified during the quiescence period). Given that drug-efflux is a common mechanism of
431 resistance in these strains, it is possible that the resistant lines reach a point at which the intracellular
432 levels of antimony decrease below a threshold required to retain the triggered quiescence phenotype.
433 It will be interesting to explore this in future work.

434 We also tested the ability of cells in which quiescence was triggered by PAT to tolerate exposure to
435 a range of other drugs. By adding 5 other compounds, with different modes of action, at their IC_{50}
436 levels, alongside PAT on day zero, we were assessing their toxic effect on non-quiescent cells. By
437 contrast, by adding the secondary drugs five days after the addition of PAT, we were testing their ability
438 to kill pre-selected quiescent cells. AMB, MIL and BOR had significantly reduced ability to clear cultures
439 if added after 5 days while this was not the case for PARO and FCCP. Mechanistically we had assumed
440 AMB might not have different impact on quiescent cells as it permeabilised membranes after binding
441 ergosterol. However, quiescent cells appear to be less sensitive to the drug, possibly indicating
442 membrane remodelling during quiescence, which might also explain why quiescent cells also showed
443 tolerance to MIL. In the case of the proteasome inhibitor BOR, the general decrease in protein turnover
444 in quiescent cells may explain why cross-tolerance is seen to this compound. FCCP interferes with
445 proton-dependent transport processes at the mitochondrial and plasma membranes and given the
446 requirement to sustain key metabolic processes the fact that quiescent cells retain sensitivity was not
447 a surprise. As an inhibitor of protein synthesis paromomycin likely continues to act on processes crucial
448 to the viability of quiescent cells, which would explain the absence of cross-tolerance to this drug.

449 Cross-tolerance to several antifungal compounds has been observed in pathogenic fungi and was
450 linked to changes in aneuploidy patterns and not the persister state [46,47]. Interestingly, it has been
451 reported by us and others that aneuploidy is very dynamic and can be adaptive in *Leishmania* [19,32,33,48,49]
452 . We analyzed genomic changes in both lines that showed cross-tolerance, but only in the recovered
453 parasites grown in drug-free medium, where no consistent chromosome copy number variations were
454 found. However, given the aneuploidy dynamics and mosaicism in *Leishmania* we cannot exclude that
455 some chromosome copy number changes contributed to the detected cross-tolerance in persister-like
456 cells.

457 In our experimental set up, the impact of transition through quiescence extends beyond the
458 duration of the non-proliferative period. We found that the lines with pre-existing adaptations to
459 antimonials (through amplification of a drug exporter and/or inactivation of AQP1 transporter)
460 emerged with reduced PAT susceptibility after recovering growth in drug-free condition. Given the lack
461 of detected genetic changes in those parasites, and the isogenic nature of the used lines we propose
462 that a form of resetting mechanism takes place, perhaps through epigenetic reprogramming that
463 explains this phenomenon. Consistent with that, Dirkx and colleagues reported previously that
464 transition through quiescence increases the parasite's infectivity and transmission potential [26].

465 The 11 *L. donovani* lines studied here exhibited here a range of quiescence and persister
466 phenotypes, which may be exploited further to probe the molecular mechanism that control the
467 transition to, maintenance and exit from quiescent state. For example, BPK087 did not exhibit
468 reduction of rEGFP expression in the very early timepoints of exposure, resulting in rapid killing. This
469 suggest that this line was not able to enter quiescence in our experimental conditions. It would be
470 interesting to see whether this remains the same if different doses of PAT are applied. If BPK087 proves
471 to be unable to enter into quiescence in response to PAT pressure, it could serve as a useful model to
472 uncover signalling that leads to quiescence entry. At the same time, several lines characterized by
473 profile 3 exhibited the ability to exit quiescence still under drug pressure and could be employed to
474 uncover the mechanisms controlling the maintenance and promoting the exit of quiescent state.

475 This study was carried out with the extracellular promastigote form of *Leishmania* and should be
476 repeated with both axenic and intracellular amastigote forms to confirm the same phenomena are
477 applicable to the clinically relevant stage. Several studies have already indicated the existence of
478 persister-like cell in *Leishmania* infections of mammals and it seems increasingly likely that *L. donovani*
479 persister-like cells can explain some cases of treatment failure in the absence of drug resistance and
480 post-kala azar dermal leishmaniasis (PKDL), observed years after clinical healing of VL.

481 In summary, this report demonstrates a worrying, intrinsic ability of *Leishmania* to tolerate high
482 lethal doses of a drug and exhibit cross-tolerance to different drugs, even with non-overlapping modes
483 of action. We also unravel unexpected effects of adoption of quiescence, which may go beyond the
484 period of exposure to drug. In strains with pre-existing genomic adaptations to the drug, transition
485 thorough the quiescent state potentially facilitates the development of a form of population-wide
486 super drug resistance. Thus, even a short period of quiescence may have much more serious impact
487 than previously thought, with a potential to create super-resistant parasites.

488

489 **Methods**

490 **Parasites**

491 Eleven strains of *L. donovani* were used in this study (Table 01). Ten were derived from clinical
492 isolates originating from VL patients in Nepal and classed into 3 main genotypes according to the
493 amplification or not of H-locus (containing MRPA) and AQP1 sequence [16] (i) No amplification of H-locus
494 and wild type AQP1 (H-/AQP+): MHOM/NP/2002/BPK026/0 clone 5 (LdBPK026),
495 MHOM/NP/2002/BPK031/0 clone 2 (LdBPK031) and MHOM/NP/2002/BPK156/0 (LdBPK156). **(ii)**
496 Amplification of H-locus and wild type AQP1 (H+/AQP+): MHOM/NP/2002/BPK080/0 clone 1
497 (LdBPK080), MHOM/NP/2002/BPK085/0 clone 8 (LdBPK085), MHOM/NP/2002/BPK087/0 clone 11
498 (LdBPK087), MHOM/NP/--/BPK190/0 clone 3 (LdBPK190), MHOM/NP/2003/BPK282/0 clone 4
499 (LdBPK282), MHOM/NP/2003/BPK294 clone 1 (LdBPK294). **(iii)** Amplification of H-locus and AQP1 indel
500 in position Ld31_0007735 (H+/AQP-): MHOM/NP/2003/BPK275/0 clone 18 (LdBPK275). A last strain
501 originating from Sudan was used as a reference: MHOM/Sudan/1962/1S-2D Bob (LdBOB). All strains
502 were cryopreserved at the Institute of Tropical Medicine in Antwerp, Belgium. Promastigotes were
503 maintained at 26°C in M199 medium (Thermo Fisher) at pH 7.2 supplemented with 20% of heat-
504 inactivated fetal bovine serum (HI-FBS), 5 mg/mL hemin, 10µM acid folic, 1X RPMI vitamin mix, 2mM
505 glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin, which was referred to as M199-
506 sup in the following experiments. For transfections, HOMEM medium (Gibco) supplemented with 20%
507 HI-FBS, referred to as HOMEM-sup, was used to transfect *Leishmania* promastigotes mentioned below.

508 **Drugs**

509 The drugs used in this study were: i) for leishmaniosis treatment: potassium antimonyl tartrate,
510 (383376, Sigma-Aldrich), miltefosine (M5571, Sigma-Aldrich), amphotericin B (A2942, Sigma-Aldrich),
511 and paromomycin (P5057, Sigma-Aldrich); ii) in development: bortezomib (5.04314, Sigma-Aldrich),
512 and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (C2920, Sigma-Aldrich). The stock
513 solutions of potassium antimonyl tartrate (PAT), paromomycin (PARO), and miltefosine (MIL) were
514 dissolved in PBS, meanwhile bortezomib (BOR), and carbonyl cyanide 4-(trifluoromethoxy)
515 phenylhydrazone (FCCP) in dimethylsulfoxide (DMSO) at a concentration of 20-50 mM and stored at -
516 20°C. The working solutions were freshly diluted in PBS or dimethylsulfoxide (DMSO) and added to
517 M199-sup until reaching the desired final concentrations for the different drugs/inhibitors. A control
518 containing either PBS (0.5% v/v) or DMSO (0.1% v/v), depending on which was used to dissolve the
519 drug or inhibitor, was included in the plate. The addition of PBS or DMSO did not interfere with the
520 parasite growth.

521 **Engineering of fluorescent lines**

522 For each strain, the enhanced green fluorescent protein (EGFP) was integrated within the 18S
523 ribosomal DNA locus (rEGFP) of promastigotes, with pLEXSY-hyg system (Jena Bioscience). rEGFP was
524 previously validated as a negative marker for quiescence: highly expressed in proliferative cells and
525 downregulated during quiescence [36]. For transfection, *L. donovani* promastigotes were maintained at
526 26°C in HOMEM-sup with passages each 4-7 days at a dilution 1/50. A quantity of 2 x 10^7 promastigotes
527 was transfected with 100 ng of the linearized DNA fragment containing EGFP, using the Basic Parasite
528 Nucleofector kit 1 (VMI 1011, Lonza); program U-033. After 24 hours of transfection, promastigotes
529 harboring rEGFP were selected with hygromycin B at a final 25 µg/ml concentration. Parasites were
530 passaged 3 times in HOMEM-sup with hygromycin B until the isolation of individual clones. An

531 appropriate dilution of 2-day promastigotes culture was washed with PBS and adjusted to a
532 concentration of 2 x 10^6 parasites/mL in PBS containing 2% HI-FBS, 100 units/mL of penicillin, and 100
533 µg/mL of streptomycin. Then, the suspension was filtered using a 5-µM pore filter to remove cell
534 clumps. A total of 8 individual clones were sorted by fluorescence-activated cell sorting (FACS), using
535 S3e TM Cell Sorter (BIORAD) using a 488 nm laser for EGFP excitation. Single cells expressing EGFP were
536 selected based on FSC-A vs SSC-A followed by FSC-A vs FSC-H, and FSC-A vs EGFP, and collected into
537 300 µL of HOMEM-sup. The clonal expansion was monitored for two weeks until enough promastigotes
538 were present for further passages. The EGFP fluorescence of the clones was assessed by FACSVerse
539 cytometer (BD Biosciences), where the clones with more than 99% of cells expressing EGFP and the
540 highest median fluorescence intensity (MFI) were selected for further experiments. The generated lines
541 were called rEGFP lines, maintained in M199-sup, and then cryo-preserved.

542 **Estimation of PAT concentration at which parasites reach a minimal metabolic activity**

543 Promastigotes of the *L. donovani* rEGFP lines were synchronized by consecutive passages every two
544 days to ensure that the promastigotes were in the proliferative phase. Then, parasites were distributed
545 (5x10^5 parasites/mL) in 200µL of M199-sup in sterile 96-well culture plates and exposed to different
546 concentrations of PAT ranging from 1200 to 12.5 µM for 4 days at 26°C. An additional well was included
547 with only M199-sup as a control for each *L. donovani* line. Resazurin sodium salt (Sigma) was added at
548 a final concentration of 80µM, and incubated for 5 h in the dark at 26°C. The fluorescence was
549 measured using the Victor X2 Multilaber Reader (PerkinElmer) with a 560 excitation and 590 emission
550 wavelength. The fluorescence measurements for each line were normalized in comparison to their
551 respective control with only M199-sup to calculate the percentage of cells with metabolic activity. The
552 lethal PAT concentrations for each line were determined as the drug concentration point where the
553 percentage of metabolic active cells was less ~1%.

554 **Monitoring quiescence and cell viability during PAT exposure**

555 The rEGFP lines were passaged twice each two days to synchronize them at the logarithmic phase
556 and then a density of 5x10^5 parasites/ml was exposed to a normally lethal PAT doses during 10 days at
557 26°C where M199-sup with PAT was refreshed once after 5 days. The respective controls were prepared
558 for each line with only M199-sup, and promastigotes were collected daily. At each time point, parasites
559 were centrifugated at 1800 x g for 10 minutes, resuspended in PBS with 1 µM NucRed Dead 647
560 (Thermo Fisher), a dead marker that binds to DNA when the cell membrane is damaged and emits
561 fluorescence. After 10 minutes at room temperature, the samples were analyzed by FACSVerse (BD
562 Biosciences) cytometer including as controls: (i) negative control, wild-type line, and not-stained, and
563 (ii) a dead control, parasites exposed to thermal shock at 70°C for 10 minutes. The gating scheme was
564 initiated with a large gate of FSC-A vs SSC-A to include all cells due to the heterogeneous size and
565 complexity of the parasites, continued with FSC-H vs FSC-A gate to avoid doublets or aggregates. A gate
566 of FSC-A vs NucRed Dead (-) was used to exclude dead cells, and live cells were determined based on a
567 gate of NucRed Dead (-) vs EGFP(+). The Flow Cytometry Standard (FCS) data files were analyzed with
568 the FlowJo™ v10.10 to determine the median fluorescence intensity (MFI) of EGFP and the percentage
569 of viable cells.

570 **Reversibility test**

571 Promastigotes from the different *L. donovani* rEGFP lines under PAT exposure during all the
572 combination and serial conditions were harvested and washed once with M199 medium by

573 centrifugation at 1800 x g for 10 minutes. The supernatant was removed without disturbing the pellet,
574 resuspended in M199-sup, and incubated at 26°C. Then, we monitored the cultures weekly for 21 days
575 under microscopic observation until flagellated mobile promastigotes were observed.

576

577 **Promastigote susceptibility assay**

578 Susceptibility of the promastigotes was assessed in the eight lines that survive PAT exposure and
579 revert to a proliferative state, including their respective controls maintained in only M199-sup medium.
580 Likewise, the promastigotes of BPK026 and BPK275 were used to measure the susceptibility against
581 five drugs for the cross-tolerance experiments. The measurement was performed using the resazurin
582 method as previously described with minor modifications [20,50]. Briefly, logarithmic-stage promastigotes
583 ($5x10^5$ parasites/mL) were plated in 200µL of M199-sup in sterile 96-well culture plates and grown in
584 the presence of M199-sup alone (control) and medium containing serial dilution of drugs for a period
585 of 72 h at 26°C. Then resazurin sodium salt (Sigma) was added at a final concentration of 80 µM, and
586 the parasites were incubated for 5 h at 26°C protected from light. The fluorescence was recorded with
587 an excitation/emission 560/590 nm using the Victor X2 Multilabel Reader (PerkinElmer). All
588 experiments were done with three independent cultures with two or three technical replicates each.
589 The IC_{50} (50% inhibitory concentration measurement) was calculated with GraphPad Prism 8 using a
590 sigmoidal dose-response model with variable slope.

591 **DNA preparation and bulk whole-genome sequencing (WGS) analysis**

592 Promastigotes were harvested from the eight lines that reverted to a proliferative state after PAT
593 exposure or controls maintained without it. The parasites were washed with PBS and the genomic DNA
594 was extracted using the QIAamp DNA Mini Kit (51306, Qiagen) following the manufacturer's
595 recommendations. The DNA concentration was measured using the Qubit dsDNA Quantification Assay
596 Kit broad-range (Q32853, Thermo Fisher). The libraries PCR-free were prepared and sequenced on the
597 Illumina NovaSeq6000 platform using 2 x 150 bp paired-end reads aiming for a sequence depth of
598 ~100x for each sample, a service provided by GenomeScan (Netherlands).

599 After quality control of the raw fastq files using FastQC, the reads were mapped to the *L. donovani*
600 reference genome LdBPK282-V2 [19], using BWA v0.7.17 using a seed length of 50. Reads with a mapping
601 quality higher than 30 were selected using SAMtools, duplicate reads removed using the Picard
602 MarkDuplicates command (version 2.22.4), and only properly paired reads were retained using
603 samtools. The somy values were estimated as the median reads count per million (CPM) per 10kb bin
604 of chromosomes normalized by the median CPM per bin of 5 chromosomes surrounding on both sides.
605 SNP calling was conducted using the Genome Analysis Toolkit (GATK) pipeline (version 4.1.4.1)
606 following GATK's best practices guidelines. The process included: (1) generating GVCF-formatted files
607 using GATK HaplotypeCaller, (2) combining the GVCF files from all samples with the GATK CombineGVCF
608 command, (3) performing genotyping with the GATK GenotypeGVCF command, and (4) filtering SNPs
609 and indels based on GATK's recommended best practices using the SelectVariants and VariantFiltration
610 commands. For the CNV detection, for every sample the sequencing depth per position was derived
611 using the SAMtools depth command. The CNV value was calculated by taking the ration of the median
612 coverage per gene over the median coverage of the chromosome the gene is located on. Statistical
613 tests comparing either CNV or Somy values were performed using a student's t-test. Visualization of
614 somy values, SNP data and CNV values was performed in R (version 4.2.0) using heatmaps and dot

615 plots. Heatmaps were created with the pheatmap package (version 1.0.12), while dot plots were
616 generated using the ggplot2 package (version 3.4.1).

617

618 **Determination of mitochondrial membrane potential (MMP)**

619 The BPK026 and BPK275 promastigotes were synchronized by two-day passaging in M199-sup
620 before each experiment. Several cultures were prepared with a density of $5x10^5$ parasites/mL with
621 normally lethal doses of PAT (50 µM for BPK026 and 200 µM for BPK275) at different times to have
622 samples under drug exposure for 2, 4, and 10 days with their respective controls with only M199-sup.
623 The mitochondrial membrane potential was determined using the MitoProbe™ $DilC_1$ (5) Assay Kit
624 (M341151, Thermo Fisher) based on the manufacturer's instructions. Parasites under PAT exposure
625 were centrifuged at 1800 x g for 10 minutes, the pellet was resuspended in M199 medium and
626 disaggregated into a single-cell suspension passing 6 times through a 26-G needle and syringe.
627 Promastigotes were centrifuged at 1800 x g for 10 minutes, washed once in PBS, and resuspended at a
628 concentration of $2 x10^6$ parasites/ml in PBS containing 100nM MitProbe $DilC_1$(5) for 45 minutes at 26°C.
629 Then, 1 µM SYTOX Blue Dead Cell stain (S34857, Thermo Fisher) was added to label dead cells for 5
630 minutes. In the experiment three controls were used: (i) a dead control, heat-killed parasites at 70°C
631 for 10 min, (ii) a positive control, parasites treated for 10 minutes with 200µM carbonyl cyanide m-
632 clorophenylhydrazone (CCCP) which disrupts mitochondrial membrane potential, and (iii) a negative
633 control, consisting of unstained parasites of a line without EGFP gene inserted. Also, fluorescence
634 minus one (FMO) controls were used to assess autofluorescence and establish the gates for the
635 selection of the cell populations. Subsequently, the samples were analyzed by FACSVerse cytometer
636 (BD Biosciences) using lasers 405nm for SYTOX Blue dye, 480nm for EGFP, and 640nm for MitoProbe
637 dye. Flow cytometric analysis was performed using FlowJo™ version 10.10.

638 **Proliferation analysis**

639 Approximately 1.5 x 10^7 promastigotes from *L. donovani* BPK026 and BPK275 were labeled with
640 CellTracker Deep Red (C34565, Thermo Fisher technologies) following the manufacturer's instructions.
641 Briefly, parasites were collected, washed once with PBS, and resuspended in 1 mL of M199 medium
642 containing 5 µM of CellTracker Deep Red. After 40 minutes of incubation at 26°C, cells were washed
643 with M199 medium, centrifuged at 1500 x g for 10 minutes, and resuspended in pre-warmed M199-
644 sup. Following labeling, 5 x 10^5 promastigotes/mL were treated with PAT at 50 µM (BPK026) and 200
645 µM (BPK275) in M199-sup or only M199-sup as a control and incubated at 26°C protected from the
646 light. The parasites were collected at three time points: 0, 4, and 10 days. At each measurement point,
647 promastigotes under PAT exposure were disaggregated using a 26-G needle and syringe and
648 centrifuged at 1800 x g for 10 minutes. The pellet and promastigotes from the controls were
649 resuspended in PBS at a concentration of 2 x 10^6 parasites/mL, and labeled with 1 µM SYTOX Blue Dead
650 Cell stain (S34857, Thermo Fisher) for 5 minutes in the dark, as a cell death marker. The fluorescence
651 data was acquired by FACSVerse cytometer (BD Biosciences) using lasers 405nm for SYTOX Blue dye,
652 480nm for EGFP, and 640nm for CellTracker Deep Red dye. Flow cytometric analysis was performed
653 using FlowJo™ version 10.10.

654 **Cross-tolerance experiment**

655 To evaluate tolerance to other anti-leishmanial drugs and drugs in development, of PAT-tolerant
656 promastigotes, we exposed the promastigotes of BPK026 and BPK275 to 5 drugs with different

657 mechanisms of action. We performed susceptibility tests by resazurin assay, mentioned above, to
658 calculate the IC50 estimates. As the promastigotes were already under high stress using normally lethal
659 PAT doses (50µM for BPK026 and 200µM for BPK275), we decided to use the IC50 estimates of each
660 drug. We designed an experimental design consisting of two exposure times of 5 days each, for a total
661 duration of 10 days where we had two conditions for each drug: (i) Combination, where the lethal
662 concentration of PAT and the drug (IC50 estimate) were present in both exposure times, (ii) Serial,
663 promastigotes were under normally lethal PAT concentration for the first exposure, followed by a mix
664 of PAT with the other drug for the second exposure. As a control group, promastigotes were under only
665 lethal PAT concentrations for both exposure times. The promastigotes were collected, centrifuged at
666 1800 x g for 10 minutes, and resuspended in M199 medium. The samples with large aggregates were
667 passed through 26-G to have single-cell suspension. The parasites were centrifuged again and
668 resuspended in PBS with NucRed Dead as a viability marker. The acquisition of the fluorescence data
669 and its gating scheme for analysis was the same as mentioned above.

670 **Statistical analyses**

671 Statistical analysis and data visualization were performed in R studio (R version 4.3.1) using
672 Student's t-test, with p-value < 0.05 considered statistically significant, and ANOVA followed by a post
673 hoc Tukey's multiple comparison test.

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Competing interests

The authors declare no competing interests.

Additional information

Supplementary material is available in Aroni-Soto_supplement.pdf

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