Genomic and metabolomic polymorphism among experimentally selected paromomycin-resistant *Leishmania donovani* strains.

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Running title: PMM resistance in Nepalase *L. donovani* strains

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Understanding the mechanism(s) underpinning drug resistance could lead to novel treatments to reverse the increased tolerance of a pathogen. In this study paromomycin (PMM) resistance (PMM-R) was induced in three Nepalese clinical strains of *L. donovani*, with different inherent susceptibility to antimony drugs (Sb), by step-wise exposure of promastigotes to PMM. Exposure to PMM resulted in the production of mixed populations of parasites even though a single cloned population was used at the start of selection. PMM IC$_{50}$ values for PMM-R parasites varied between 104-481 µM at the promastigotes stage and 32-195 µM at the intracellular amastigotes stage. PMM resistance was associated with increased resistance to nitric oxide at the amastigote but not the promastigote stage (p < 0.05). This effect was most marked in the Sb-R PMM-R clone, where PMM-R resistance was associated with a significant upregulation in glutathione compared to its WT (p < 0.05) although there was no change in trypanothione (detected in its oxidised form). Interestingly, PMM-R strains showed an increase in either the keto acid derivative of isoleucine (Sb-I PMM-R) or the 2-hydroxy acids derived from arginine and tyrosine (Sb-S PMM-R and Sb-R PMM-R). These results are consistent with the recent finding that upregulation of the branch-chain amino acid aminotransferase and the D-lactate dehydrogenase are linked to PMM-R. In addition, we found that PMM-R was associated with a significant increase in aneuploidy during PMM selection in all the strains, which could allow rapid selection of genetic changes that confer a survival advantage.
INTRODUCTION

Visceral leishmaniasis (VL), a disease caused by infection with the protozoan parasites Leishmania donovani and L. infantum (syn. L. chagasi) is fatal if left untreated. There are 360 million people at risk of infection and in 2017, global incidence was estimated between 50,000 and 90,000 new VL cases each year, with most of them occurring in just six countries (1). Co-infection with HIV is associated with exacerbated disease and a poorer chemotherapeutic prognosis (2). Disease control is primarily focused on vector control and drug treatment of clinical cases as there is no vaccine to protect people against infection. There are a limited number of drugs to treat VL and treatment failure and/or drug resistance is jeopardizing their efficacy as already illustrated in the cases of antimonials (Sb, 3-4) and miltefosine (MIL, 5). In the expectation of new compounds, alternative drugs such as amphotericin B (or its liposomal formulation, AmBisome®) as well as combination therapy are being used as first-line treatment in some endemic areas. Paromomycin (PMM) is an aminoglycoside antibiotic shown to have activity against Leishmania that is increasingly tested in combination regimens (6). In Bangladesh, in combination with MIL or AmBisome®, PMM showed a similar efficacy as AmBisome® monotherapy for the treatment of VL (7). A study has shown that combined sodium stibogluconate (SSG) and PMM treatment in Sudan, Kenya, Uganda and Ethiopia was an effective first line option for VL chemotherapy (8). A retrospective study on treatment of post-kala azar dermal leishmaniasis (PKDL), a complication of VL, showed that combination therapy with PMM was more effective than monotherapy with SSG in East Africa (9).
Experimental work showed that selection of PMM-resistance in *L. donovani* was rather easy and fast (10, 11). Given the increased usage of PMM in clinical practice, it is thus important to understand the molecular mechanisms underlying PMM-resistance in *Leishmania*. The drug is known to down-regulate protein synthesis, and it has been shown to affect multiple protein targets in *L. donovani* (12). The cytoplasmic target of PMM is the decoding A-site of the small subunit (SSU) of ribosomes, where it increases misreading and translation inhibition. Steric hindrance caused by a 1408A to G mutation at the gene region encoding the 18S ribosomal RNA (rRNA) helix h44, which forms part of the A-site or the rRNA, was thought to be responsible for the poor binding of PMM to eukaryotic cytosolic ribosomes. Mutations at positions 1408 and 1491 are known to be important in determining sensitivity to PMM (13). A study using a high throughput Cosmid Sequencing (Cos-Seq) approach identified genetic changes on 5 chromosomes that were conferring PMM-resistance in *L. infantum*. However, the product of these gene sequences was unknown and Cos-Seq could not identify loss of function targets (14). Previous work showed that molecular mechanisms of drug resistance may vary with the genetic background of the parasite (15). In present study, we thus selected PMM resistance (PMM-R) in three *L. donovani* clinical strains from Nepal, with different susceptibilities to Sb drugs and different genetic backgrounds. The wild-type strains were previously phenotyped as Sb-sensitive (Sb-S), Sb-intermediate (Sb-I), or Sb-resistant (Sb-R), respectively (16). We characterized the effect of PMM-R on the metabolome of the strains as well as their infectivity and susceptibility to
antimicrobial products produced by macrophages. We sequenced the whole genomes of the Sb-S and Sb-R strains during induction of PMM-R. We show polymorphic molecular changes among the different PMM-R strains.

RESULTS

PMM-susceptibility upon adaptation of Sb-S, Sb-I and Sb-R lines to PMM

The Sb-S WT parent used here (BPK280/0cl4) was previously found to be intrinsically less susceptible to PMM than the other two *L. donovani* strains used in this study (Table 1). However, this strain was submitted to the same PMM-resistance selection protocol to determine if PMM selection resulted in increased PMM resistance as studies have shown that drug resistant parasites can lose their drug resistance if drug pressure is removed (17). After adaption of the parasites to grow in 97 µM PMM, promastigotes of the induced Sb-S PMM-R parent line had a lower IC$_{50}$ value compared to the WT but cloned lines from this parent either had a lower or similar IC$_{50}$ value as the original WT after PMM selection (Table 1, data for one cloned line shown). This may indicate that there is an upper limit on the ability of this strain to tolerate PMM exposure and that selection resulted in the expansion of different subclones from the original WT. At the amastigote stage the Sb-S PMM-R parent and cloned line had a similar or lower susceptibility to PMM (Table 1). In contrast selection using PMM resulted in increased resistance to PMM at both the promastigote and amastigote stage for the Sb-I strain (BPK087/0cl11) and Sb-R strain (BPK275/0cl18). The PMM-R cloned line from the PMM-R Sb-I or PMM-R Sb-R parent had a similar susceptibility to PMM (Table 1).

Metabolomic profile of PMM-R parasites
Metabolites of the three *L. donovani* PMM-R clones i.e. Sb-S PPM-R clone 8, Sb-I PMM-R clone 8 and Sb-R PMM-R clone 9 and their corresponding WT were extracted on the same day of culture from parasites that grew at the same rate, and analysed to identify metabolomic changes associated with PMM resistance. The same 212 metabolites were putatively identified in all six lines (see Supplemental Excel files 1 and 1a at https://doi.org/10.15129/88f47c26-ff93-4a7c-8c17-d323aa24052e). PCA analysis showed that replicates of each of the *L. donovani* strains clustered into separate groups (Fig. 1a). Clear differences were obvious when each WT was compared to its corresponding PMM-R derivative using OPLS-DA (Fig 1, b-d) indicating that clear metabolic differences occurred on adaption to 97 μM PMM. Overall there were more differences in Sb-I PMM-R and Sb-R PMM-R than in Sb-S-PMM-R (Supplemental Tables S1 and S2 at https://doi.org/10.15129/88f47c26-ff93-4a7c-8c17-d323aa24052e). Fig 2 shows a heatmap of the relative expression of metabolites shown in Table S1, to highlight the effect of induction of PMM-R on the metabolomic profile of parasites. Metabolic changes occurred in a number of biochemical pathways i.e. amino acid metabolism, glutathione metabolism, carbohydrate metabolism, glycolysis, the TCA cycle and nucleotide metabolism. It was possible to relate some changes to specific biochemical pathways within pairs of strains. For example, *Leishmania* species produce hydroxy acid derivatives of aromatic amino acids and arginine (18). This process requires production of a 2-keto acid by transamination of the amino acid followed by reduction of the keto acid to form the corresponding 2-hydroxy acid (Fig 3a) and specific products for arginine, tyrosine and tryptophan were detected in the metabolic
profile (Fig 3b,c). Significant differences were observed in arginine metabolites, with a large decrease in 2-oxoarginine levels and a corresponding increase in arginic acid for the Sb-R PMM-R clone compared to its WT (Fig 3c). The same trends were found for the Sb-S PMM-R clone, although the changes were smaller and generally below the 2-fold threshold. In contrast the Sb-I PMM-R clone had significantly higher levels of the keto acid derivatives of arginine and tyrosine (2-oxoarginine and Hydroxyphenyl pyruvate (HPA), respectively) but no significant changes in the corresponding 2-hydroxy acids (arginic acid and HPLA). The changes in 2-hydroxy acids indicates increased 2-hydroxy acid dehydrogenase, although the metabolic enzymes responsible for this activity have not been identified in *Leishmania*. This activity could be important for NAD/NADH recycling to maintain redox balance within the promastigote, so the change could be an indirect effect of PMM-R. Levels of tryptophan varied between the strains but there was no corresponding difference in its hydroxy acid derivative indolelactate. Arginine is involved in a number of metabolic pathways (Figure 4), and could affect proline levels. Proline has been shown to protect against oxidative stress and its upregulation has been associated with drug resistance (18). Proline levels were upregulated in the Sb-I PMM-R clone compared to its WT but not the other two PMM-R clones. Therefore, in order to determine if proline could mediate PMM resistance in the Sb-S and Sb-I strains, the WTs and their corresponding PMM-R clones were exposed to PMM at different concentrations in medium supplemented with proline. Addition of proline to the medium had no significant effect on parasite growth or response to PMM treatment, giving further evidence that proline was not involved in PMM-R.
Trypanothione, the main intracellular thiol and reducing agent in trypanosomatids, was detected (in its oxidised form) but there were no differences observed between WT strains and their PMM-R derivatives (Fig 4). It should be noted that thiols can be oxidised in-situ during LC-MS and the levels observed are indicative of the total amount in the extract rather than their oxidation state in vivo. Other compounds can also protect against oxidative stress and in the Sb-R PMM-R clone there was a significant upregulation in the levels of glutathione (p < 0.05). However similar metabolic changes were not present in the other two PMM-R clones (Fig. 4).

PMM-R resistance had little effect on glucose metabolism of parasites as majority of metabolites in the TCA cycles were unaffected compared to WTs (Fig. S1). This could indicate that PMM-R does not require active transport mechanisms that require energy.

**Lipidomic profile of PMM resistant parasites**

None of the lipids identified in lipidome studies were significantly altered after PMM-R selection for the Sb-I PMM-R strain compared to its WT (Table S2). In contrast, 7 lipids were significantly down-regulated and 1 lipid (sphingenine) was up-regulated in Sb-R PMM-R compared to its WT. Of these 7, four lipids were small, unsaturated PEs (two diacyl PEs, one alkyl-acyl PE and one alkenyl-acyl PE). The other three were a ceramide, a PI and an LPE (Table S2). In the Sb-S PMM-R, there was a significant upregulation of 4 alkyl-acyl phosphatidylinositols. There was also a significant reduction of 4 other lipids that are not structurally related.

**PMM-R does not affect infectivity but influences macrophage responses**
In previous studies of MIL-resistant (MIL-R) parasites derived from the same WT parents, we found that MIL-R was associated with a reduction in macrophage infectivity (19). Therefore, studies were carried out to determine if PMM resistance affected parasite ‘fitness’ by assessing their ability to infect macrophages and their susceptibility to antimicrobial products produced by macrophages. PMM-R was not associated with a reduction in infectivity to macrophages as parasite levels were similar in macrophages infected with WT or PMM-R parasites (data not shown). Sb-S PMM-R parasites had a significantly greater resistance to IFN-γ/LPS induced killing (p < 0.05), but only if cells were treated with the lower dose of IFN-γ/LPS (i.e. 50 units IFN-γ/50 ng LPS/ml vs 100 units IFN-γ/100 ng LPS/ml, data not shown). The Sb-I and Sb-R PMM-R parasites did not demonstrate a consistent difference in susceptibility to IFN-γ/LPS induced killing compared to their corresponding WT at either dose (data not shown. Treatment with SNAP, a nitric oxide (NO) inducer, caused a dose-dependent reduction in parasite survival, and the effect was not related to any difference in NO production as similar amounts of nitrite were present in supernatants of uninfected and infected macrophages treated with SNAP (data not shown). Sb-S WT and Sb-S PMM-R promastigotes had similar tolerances to SNAP-induced parasite killing whereas Sb-I PMM-R and Sb-R PMM-R clones were less resistant to NO exposure compared to their respective WT (Table 2). At the intracellular amastigote stage all independently cloned Sb-S promastigotes tested except one, Sb-S PMM-R clone 6, were significantly more resistant to SNAP treatment compared to their WT counterpart (p< 0.05, only data for clone 8 shown in Table 2). The anomaly for the Sb-S PMM-R clone 6 may be
because this clone had the same PMM susceptibility as the Sb-S WT (data not shown). The Sb-R WT exhibited the highest resistance to SNAP treatment at the promastigote stage of the three WTs, with an IC$_{50}$ value that was 9 times higher than the other two WTs. This may reflect the inherent resistance of this strain to Sb as Sb-R is related to enhanced resistance to RNS (20). This may explain why the increase in SNAP resistance for the Sb-R PMM-R clone 9 at the amastigote stage is only 1.3 times higher than the Sb-R WT, whereas the other PMM-R parasites expressed a much higher differential resistance to SNAP treatment (relative resistance to WT: Sb-S PMM-R/Sb-S WT 2.1-2.6 times higher, Sb-I PMM-R/Sb-I WT 25.3 times higher). SIN-1 treatment, which induces both NO and superoxide production in macrophages, did not give a clear differentiation in the ‘fitness’ of WT and PMM-R parasites based on the IC$_{50}$ value for SIN-1 exposure (Table 2). Sb-S PMM-R and Sb-R PMM-R parasites had a similar susceptibility to SIN-1 as their corresponding WTs. Whereas, Sb-I PMM-R clone 8 promastigotes were significantly more susceptible to SIN-1 treatment than the Sb-I WT ($p < 0.05$). There was a difference in SIN-1 susceptibility between the WT, with the Sb-I WT being the most resistant and the Sb-R WT being the least resistant (Table 1). Overall the most striking phenotype associated with PMM-R was increased resistance to NO measured at IC$_{50}$ value for SNAP exposure in the amastigote stage of the parasite. The Sb background of the strains did have an influence, with the Sb-R PMM-R strain exhibiting greater resistance to NO.

**Genetic variants discriminate PMM-S from PMM-R lines**

Only the Sb-S and Sb-R strains were used in genotyping studies to determine what influence the two extreme phenotypes of Sb susceptibility had on PMM-
We screened genome-wide data for mutations associated with PMM tolerance at the WT, 2, 4, 8 and 97 µM PMM-R stages for both Sb-S and Sb-R, along with 32 and 64 µM for the Sb-R line. The Sb-S sample had a dose-dependent aneuploidy signature: the copy number of chromosomes 6, 7, 8, 13, 14, 15, 22 and 32 (eight in total) increased from disomy in the WT to trisomy in both Sb-S PMM-R libraries at 8+ µM PMM (Fig. 5). The Sb-R chromosome copy numbers fell to disomy from trisomy for chromosomes 11 and 14 (at 32+ and 64+ µM PMM respectively), as well as from tetrasomy to trisomy for chromosomes 2 and 33 (at 32+ µM PMM) but rose from disomy to trisomy for chromosomes 1 and 5 (at 32+ µM PMM). Consequently, there was a marked difference in the somy-level response for the Sb-S and Sb-R isolates, highlighting that their adaptation to this drug may differ.

Transient amplification of the rRNA locus at both 1,046-1,053 and 1,058-1,065 Kb on chromosome 27 were discovered in both Sb-S and Sb-R isolates at 2, 4 and 8 µM PMM, but this was absent in the WT and 32/64/97 µM PMM lines (Text S2, Figures S2 and S3). These regions spanned three (LdBPK_270030120-140) and four (LdBPK_270030190-220) genes, respectively, where the first gene of each amplification were the 18S SSU rRNA genes. This increased the local rRNA gene dosage from six (WT) to ten (2-8 µM PMM), but this reverted back to six in the 32-97 µM PMM.

The remaining other genetic changes were a deletion and a heterozygous SNP. The sole deletion observed was in Sb-S PMM-R isolates, and was a heterozygous loss of a 1,532 bp region containing a NAD-dependent epimerase/dehydratase family gene. The sole consistent SNP was a heterozygous (G>A) one at a mitogen-activated protein kinase kinase
kinase (MAPKKK) gene (LdBPK_190007100) at chr19:45,638 in the Sb-S isolate with a low (0/2/4/8 µM PMM) dose that became fixed in both Sb-S PMM-R isolate clones (97 µM). No SNPs were found in the Sb-R lines during PMM exposure. No other PMM-related SNPs, insertions or episomes were observed that distinguished all PMM-S from all PMM-R lines in either the Sb-S or Sb-R isolates.

DISCUSSION
The results indicated the induction of PMM-R parasites from a single cloned WT can result in a mixed population of parasites, based on the response of individual Sb-S PMM-R sub clones and the parental drug-resistant promastigotes parasites to SIN-1 or SNAP treatment. The PMM IC₅₀ value for Nepalese *L. donovani* WT at the start of this study (Sb-S PMM-R, 354 ± 4 µM; Sb-I PMM-R 56 ± 22 µM; Sb-R PMM-R, 65 ± 2 µM), were higher than those quoted for *L. aethiopica* clinical isolates from patients with localised cutaneous leishmaniasis (4.44 µg/ml or 7.22 µM), mucosal cutaneous leishmaniasis (21.8 µg/ml or 35.45 µM) or diffuse cutaneous leishmaniasis (0.20 µg/ml or 0.33 µM, 21).

One of the most striking phenotypes associated with PMM-R parasites compared to their WT was the increased resistance to NO, which was expressed primarily at the intracellular amastigote stage, and the Sb-S PMM-R clone had a lower resistance compared to the Sb-I PMM-R and Sb-R PMM-R parasites. The higher resistance to NO probably reflects adaptations related to Sb-R, which also gives increased resistance to NO (22). A relationship between PMM-R and increased resistance to nitrosative stress has been reported in *L. infantum* at the intracellular amastigote stage (11) and
an Indian *L. donovani* PMM-R clinical isolate (BHU573), that had PMM-R induced using the same protocol, showed increased resistance to SNAP treatment at the promastigote stage but a significantly lower tolerance at the intracellular amastigote stage (23). It is possible that clinical use of PMM as antibiotic treatment rather than a first line antileishmanial treatment may have favoured the survival of naturally occurring NO resistant parasites. There is a precedent for this type of phenomenon as genotyping of historical bacterial strains indicate that methicillin-resistance was a result of using methicillin to circumvent penicillin resistance in the 1940’s. The limited use of methicillin then provided the selective pressure to drive the nosocomial spread of a variant that was present in the bacterial population (24). A natural isolate of *L. chagasi* had a SNAP IC$_{50}$ value of 48.8 mM, which is approximately 100 times higher than the IC$_{50}$ obtained in this study, but the PMM sensitivity of this strain was not determined (25). There is data to indicate that selection at the amastigote stage can have a more profound effect on PMM susceptibility in *L. donovani*. In this study adaptation to PMM-R to 97 µM took 26 weeks to develop when using the promastigote stage and studies using the AG83 *L. donovani* strain found that it took 3 months to select PMM-R using a 10 µM stepwise exposure of promastigotes to PMM, up to a maximum of 200 µM. In contrast, Hendrickx *et al.* (10), who used the same Sb-R WT parent (BPK275/0cl8), found that resistance to PMM could be induced within 2 selection cycles if the intracellular amastigotes were exposed to PMM (IC$_{50}$ µM ± SD, 199.0 ± 8.5). This may indicate that selection of PMM-R can occur more rapidly if drug selection occurs at the amastigote stage. Therefore, it would be prudent to monitor the resistance of clinical strains to all clinically
used anti-leishmanial drugs to screen for the evolution of 'multiple drug resistant' strains. The metabolic and lipidomic changes in the three PMM-R types of parasites produced in this study were different and strain-specific, suggesting that PMM-R was mediated by different mechanisms in strains with different genetic backgrounds. There was no clear marker for the PMM-R from an untargeted analysis of metabolites present or a targeted analysis determining metabolites associated with resistance to oxidative stress in Leishmania, i.e. glutathione and proline. Surprising fewer metabolic/lipidomic changes occurred for PMM-R compared to MIL-R induced using the same parent clones (19). This may be related to the mode of action of these drugs, indicating that PMM has fewer targets in the parasite compared to MIL. This finding is similar to the results of Berg et al. (17) using an L. donovani strain isolated in Ethiopia (MHOM/ET/67/HU3) where PMM-R resulted in 14 metabolic changes compared to the WT whereas MIL-R induced 20 changes. A recent study found that PMM-R resistance was associated with increased glycolytic activity to form products that can scavenge hydrogen peroxide (26). However, results from this study did not show any major changes in TCA cycle metabolites. PMM-R was associated with changes in 2-hydroxy acid production from certain amino acids. In Leishmania transamination of amino acids to their corresponding 2-keto acids involves a broad specificity amino transferase that can use aromatic amino acids and a branch-chain amino acid aminotransferase (BCAT) with a specificity for leucine, isoleucine and valine (27). The 2-hydroxy-acid dehydrogenase(s) responsible for reduction of the 2-keto acids to form lactates has not been identified. In lactic acid bacteria
and protozoa such as *Trichomonas vaginalis* these reactions are catalysed by the canonical NAD-dependent hydroxy acid dehydrogenase, lactate dehydrogenase (LDH), an enzyme with broad substrate specificity (30). *Leishmania* species do not encode LDH but genes for malate dehydrogenases (MDH) and a putative D-lactate dehydrogenase (D-LDH) are present. Recently, up-regulation of D-LDH and BCAT mRNA was linked to PMM-R resistance in *L. donovani* by RNA-seq analysis of WT and PMM-R strains (15). Over-expression of the putative D-LDH in *L. donovani, L. major* and *L. infantum* also increased PMM resistance and there was cross-resistance with other aminoglycosydic antibiotics. Similar results were observed following over-expression of BCAT although there was no additive effect when BCAT and D-LDH were co-expressed in the same line. The authors proposed that enzymatic modification of amino or hydroxyl groups on the antibiotics could contribute to the resistance mechanisms of BCAT and D-LDH (15). In the present study, levels of 2-keto-3-methylvaleric acid (KMVA), the 2-keto acid derivative of isoleucine indicates increased BCAT activity in Sb-I PMM-R compared to the corresponding WT. High levels of HPHA and arginic acid in the PMM-R strains derived from Sb-S and Sb-R must result from increased 2-hydroxy acid dehydrogenase activity. This new metabolomic data supports a role for increased BCAT and or D-LDH activity in PMM-R resistance in *L. donovani*, based on RNAseq analysis.

Aneuploidy is hypothesised to represent an early adaptive response to drugs but this assertion is complicated by its intrinsic dynamic nature of within and across *Leishmania* species and life cycle stages to modulate the expression of the majority of genes (31, 32). Here, the higher copy number of
seven chromosomes for the Sb-S isolates associated with 8+ µM PMM contrasted with the jump in somy for different chromosomes in the Sb-R (1 and 5), along with the fall in somy for chromosomes 2, 11 and 33 at 32+ µM PMM and also for chromosome 14 at 64+ µM. This result shows again that the genetic background of the strains used in the selection experiment may influence the outcome of the consecutive molecular adaptations. This genetic diversity may also explain why PMM selection resulted in the emergence of PMM-R parent and subclone with different PMM IC$_{50}$ values from the Sb-S WT. A study comparing the efficacy of dosing _L. donovani_ patients in Sudan with 15 mg/kg PMM for 28 days with PMM at 20 mg/kg for 21 days showed that peak PMM plasma levels occurred approximately 1-2 hours after dosing, and that both dosing regimens gave drug levels that were much lower than 13 µg/ml (mean values on day 1, 20 mg/kg, 7.8 ± 4.9 µg/mL i.e. 12.6 µM; 15 mg/kg, 5.6 ± 4.2 µg/ml i.e. 9.1 µM). At 6 months follow up both of these regimens were associated with 80-81 % cure rate, indicating that some patients still harboured parasites (33). Based on our data the remaining parasites would have been exposed to sufficient drug (i.e. > 8 µM) to induce change in gene somy required for PMM-R.

PMM is an aminoglycoside, effecting ribosomes function and membrane fluidity (23) and studies have shown that PMM targets the decoding A-site of the ribosome SSU, with mutations at positions 1408 and 1491 determining sensitivity to PMM (13). In this study, we found a transient rRNA locus amplification at 2-8 µM PMM in both Sb-S and Sb-R, indicating that at low doses the 18S SSU gene transcription rate probably increases, highlighting an alternative PMM tolerance mechanism. The only consistent
SNP in PMM-R parasites was a heterozygous (G>A) one at a MAPKKK gene in the Sb-S line. MAPKKK proteins activate MAPKK proteins through the phosphorylation of serine and serine/threonine residues in its T-loop. A study in *Larix olgensis* found that MAPKKKs were induced by NO at the transcriptional level, and it is known that NO upregulates MAPKs (34). So perhaps it is no coincidence that in this study PMM-R in *L. donovani* was associated with increased resistance to NO induced by SNAP treatment. This is important to ascertain as it may be possible to produce an inhibitor or provide a supplement that could reverse PMM resistance and extend the clinical life of the drug against *L. donovani*. In a previous study based on Cos-Seq, the copy number of LinJ.06.1010 gene, with its leucin-rich repeat domain was found to be responsible for the resistance to PMM (35), but this was not verified by Rastrojo *et al.* 2018 (36), while in present study, we did not find local copy number variation, well an increase of chromosome 6: these different results obtained on different strains might again highlight the influence of the genetic background on the outcome of molecular adaptations developed during resistance selection. Last but not least, our work is consistent with the lack of PMM-MIL cross-resistance (38) underlining the potential use of this pair as a combination treatment.

In summary, this study showed that resistance to PMM was present in an *L. donovani* isolate from Nepal in the absence of PMM drug pressure. It was relatively easy to induce resistance in *L. donovani* and the sub clones produced from the parental PMM-R line can express different levels of PMM resistance. PMM-R was associated with enhanced resistance to NO and the mechanisms that control PMM-R varied between lines, and the Sb resistance
profile of the parasite together with its genetic background had an impact on the outcome of selection. The PMM-R strains had significantly higher levels of either the keto acid, KMVA, or 2-hydroxy acids (arginic acid and HPLA) that could indicate upregulation of BCAT or D-lactate dehydrogenase, activities recently linked to PMM-R. The challenge now is to expand this study to a larger number of field isolates, to determine what is the predominant phenotype present in endemic areas.

MATERIALS AND METHODS

Reagents

Pure crystalline PMM (paromomycin-sulphate USP) was supplied from Gland Pharma, India. Resazurin, SNAP, Giemsa stain, and lipopolysaccharide were purchased from Sigma-Aldrich (Gillingham, UK). HOMEM medium, RPMI-1640, PBS pH 7.4, penicillin/streptomycin, glycine and foetal calf serum were obtained from Invitrogen, Paisley, UK. SDM medium was custom made by Gibco, Paisley, UK. S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) and 3-morpholinosydnonimine (SIN-1) were purchased from Enzo Life Sciences, Exeter UK. All other reagents were on analytical grade.

Animals and parasites

Age-matched inbred BALB/c female mice (20-25 g) were used in studies at Strathclyde University. Animal studies were carried out with local ethical approval and had UK Home Office approval. *L. donovani* cloned strains with different Sb susceptibility backgrounds were derived from isolates obtained from VL patients at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal: MHOM/NP/02/BPK282/0cl4 (Sb-S). MHOM/NP/02/BPK087/0cl11 (Sb-intermediate, Sb-I) and MHOM/NP/02/BPK275/0cl18 (Sb-R, 16).
Selection of PMM resistance (PMM-R) clones

*L. donovani* strains were adapted to grow in a step-wise manner in increasing concentrations of PMM (2, 4, 8, 16, 32, 64, 97 µM) until all lines grew at similar rates as wild-type (WT) parasites in HOMEM medium supplemented with 20% v/v foetal calf serum (complete HOMEM). The highest concentration of PMM used was 97 µM as parasites did not grow well in higher drug concentrations. Parasites selected to grow as well as WT in 97 µM PMM were termed ‘PMM-R’ and their specific designation depends on the WT used i.e. Sb-S PMM-R parental line was derived from the BPK282/0cl4, the Sb-I PMM-R was derived from the BPK087/0cl11, and Sb-R PMM-R was derived from the BPK275/0cl18. Clones from the parental drug resistant line were isolated, expanded in Locke Medium on top of Tobie’s agar base, and then grown in complete HOMEM. A stock solution of aqueous PMM (3 mg/ml, freshly prepared every 3 months) was stored at -20°C and used to prepare drug selection medium. Clones were produced from the parental PMM-R lines using the micro drop technique (10) and designated with a number.

Genetic characterisation of Sb-S PMM-R and Sb-R PMM-R strains

Cell harvesting, lysis, genomic DNA isolation and genome sequencing was completed as outlined previously (19). Genomic DNA was sequenced on Illumina HiSeq 2000 platforms, with a median sequence coverage of 47.4 ± 17.8 reads per site averaged across 15 sequence libraries (Supplemental Table S2 at https://doi.org/10.15129/88f47c26-ff93-4a7c-8c17-d323aa24052e). Following screening for contamination, DNA reads for each library with insert sizes of less than 1,000 bases were mapped to a new *L. donovani* LdBPK282 reference genome using Smalt with exhaustive
alignments (v0.7.4, https://sourceforge.net/projects/smalt/, 38). All non-mapping and PCR duplicate reads were removed. Sites with low quality scores; or in repetitive regions; or with poor mapping scores; or in low-complexity regions; or with significant forward-reverse strand amplification bias; or with low read coverage were excluded. Chromosome copy number variation and scans for large CNVs and episomes were performed based on per cell read depth to reflect gene dosage as outlined previously (39). Changes in chromosome copy numbers were detected by comparing the distribution of coverage levels across all sites for each chromosome using t-tests as outlined elsewhere (37). SNPs, small insertions and deletions were called using population-based Unified Genotyper method in Genome Analysis Toolkit v3.4 with parameters described previously (37).

**Phenotyping of promastigotes**

The effects of amphotericin B (AMB), PMM, SIN-1, SNAP on the growth of promastigotes were determined using a colorimetric-based assay following the method described by Shaw et al. (19). Parasites (5 x 10^5 parasites/100 µl, n = 6/treatment) were exposed at time 0 to AMB (1 µM), various concentrations of PMM, SIN-1 or SNAP (n = 6/treatment) for one hour (SIN-1 studies) or 72 hours (AMB, PMM or SNAP studies) at 27°C. After 1 hour exposure the SIN-1 solution was removed, fresh medium added and cells were incubated for a further 71 hours. In some studies proline (200 mM) was added to the medium with and without PMM solution, as this proline concentration was used in previous studies investigating the role of proline in drug resistance in *L. donovani* (17). Parasites were exposed to AMB to show whether resistance to PMM was drug-specific. Resazurin solution (20
µl/sample, 0.0125% w/v PBS pH 7.4) was added to samples for the last 18 hours of culture and then the absorbance of samples at 550-590 nm was determined using Softmax Pro 2.0 software. The effect of drug treatment on cell viability was determined by calculating the mean suppression compared to the relevant mean control value, and this was used to determine the IC₅₀ for a particular drug/compound using Grafit® 5 software.

**Metabolic and lipidomic characterisation studies**

Extraction of the lipidic or metabolites was carried out using 4 x 10⁷ promastigotes L. donovani promastigotes/strain obtained on day 4 of culture using the methods described by Shaw et al. (19). Specific metabolites were identified using IDEOM and 4 standard mixes were used in assays, whereas lipids were identified using Xcalibur (version 2.2) and an in-house Excel macro. PCA and OPLS-DA plots were produced using published methods (40) and SIMCA Software (version 14.1). Results were repeated three times but data from one single representative experiment using all samples in one run are shown.

**Phenotyping of intracellular amastigotes**

The method described by Carter et al. (22) was followed. Briefly, peritoneal macrophages (0.5 x 10⁵/sample, n = 5/treatment) from BALB/c female mice were infected with L. donovani promastigotes at a host: parasite ratio of 20:1 for 24 hours. Infected cells were incubated for 72 hours with medium alone (controls), SNAP (10-160 µg/ml) or IFN-γ, LPS (IFN- γ, 50 or 100U/ml; LPS, 50 or 100 ng/ml). The percentage of infected macrophages and the mean number of parasites/host cell were determined from 200 randomly selected infected cells; infectivity was determined by multiplying both parameters. The
effect of treatment on parasite survival was determined as the mean percentage suppression compared to the relevant mean control value. Experiments were repeated a minimum of three times.

**Statistical analysis of data**

The effect of drug treatment on cell proliferation in *in vitro* drug studies were analysed using a Mann Whitney U test for comparing two treatments or a Kruskal Wallis test followed by Dunns ad hoc test for statistical differences between three or more treatments (Statview® version 5.0.1 software package). Results were considered significant at a p value < 0.05.

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**FIGURE LEGENDS**

**Figure 1. Multivariant analysis of data from LC-MS.** Multivariant analysis of data from LC-MS was performed using SIMCA. There are 214 features (metabolite peak intensity values) per sample. Plots show scores resulting from modelling of data matrix: A, PCA-X analysis of all samples; B, OPLS-DA of Sb-S strains; C, OPLS-DA of Sb-I strains; D, OPLS-DA of Sb-R strains. Elipses represent 95% tolerance limits (Hotelling’s T2, 38).
Figure 2. The effect of PMM selection on the metabolic profile of PMM-R L. donovani promastigotes compared to WT. Heat maps of metabolite levels (mean peak intensity) were generated using Metaboanalyst 4.0. The map shows mean metabolite levels for the different L. donovani strains (columns) based on mean peak intensity values from LC-MS (rows). Deep red represents the highest level and deep blue represents the lowest level, with white representing equal levels. Each group is identified by a coloured bar at the top of each column and by the name of the strain at the bottom. This data is representative of three separate experiments.

Figure 3. Hydroxy acid production in the different L. donovani strains. a, General pathway for production of hydroxy acid derivatives of amino acids in Leishmania (a). 2-Keto acids and 2 hydroxy acids produced from isoleucine, arginine, tyrosine and tryptophan (b). Differences in hydroxy acid levels in wild type and PMM resistant strains (Log2–fold change in metabolite peak intensity, c). * P ≤ 0.05. Abbreviations: KMVA, 2-keto-3-methylvaleric acid; HMVA, 2-hydroxy-3-methylvaleric acid; HPP, Hydroxyphenylpyruvate; HPLA, Hydroxyphenyllactate.

Figure 4. Arginine and methionine metabolism in different strains of L. donovani. Pathway map for arginine, proline, methionine and cysteine metabolism. The map also includes glutathione and trypanothione biosynthesis. Boxed metabolites were detected in metabolic profile of the L. donovani strains by LC-MS: yellow shading indicates that metabolite identities confirmed by matching retention times with those obtained with authentic standards, unshaded metabolites were putatively identified by accurate mass only. Unboxed metabolites were not detected by LC-MS but are presumed to
be present. Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; MTA, 5-methylthioadenosine; OAS, O-acetylserine; GSH, glutathione; GSSG, oxidised glutathione; T(SH)2, reduced trypanothione; T(S)2, oxidised trypanothione; P5C, Pyrroline-5-carboxylate.

Filled arrows represent enzyme reactions known to occur in *Leishmania* spp. Dotted arrows show reactions that have not been confirmed. *a*The urea cycle is not thought to be complete in *Leishmania*. *b*Nitric oxide synthase activity was detected in *L. amazonensis* but the gene has not been identified in any *Leishmania* species. *c*The oxidation of thiols may occur *in situ* during cell extract formation and LC-MS.

**Figure 5.** Differing aneuploidy stress responses during PMM selection of the Sb-S (BPK282/0cl4) and Sb-R (BPK275/0cl8) lines. The heatmap shows the copy-number status of the 36 chromosomes from 0 (wild-type) to 2, 4, 8, 32, 64, 97 µM PMM as disomic (blue), trisomic (green) or tetrasomic (ivory). The colour key shows the normalised chromosome read-depth and the distribution frequency. Chromosomes 6, 7, 8, 13, 14, 15, 22 and 32 (all at 8+ µM) shifted from disomy to trisomy in Sb-S but not Sb-R, which showed an increased somy for chromosomes 1 and 5 (at 32+ µM), but lower somy for chromosomes 2, 11 and 33 at 32+ µM and 14 at 64+ µM.

**Table 1.** The relative susceptibility (IC50) of different *L. donovani* strains to PMM. The data shows the means IC50 from a minimum of three separate experiments. *p < 0.05 compared to corresponding WT.

**Table 2** The susceptibility of (IC50) different *L. donovani* strains to SIN or SNAP treatment. The mean IC50 values are from 3 separate experiments. *p < 0.05, compared to respective WT.
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**Data availability**

Supplemental data and data underpinning this publication are available from the University of Strathclyde KnowledgeBase at [https://doi.org/10.15129/88f47c26-ff93-4a7c-8c17-d323aa24052e](https://doi.org/10.15129/88f47c26-ff93-4a7c-8c17-d323aa24052e). Raw sequence reads are available from the European Nucleotide Archive (ENA) via accession number ERP115194 and individual sample accession numbers are listed in Table S4.
Fig. 1
Fig. 2
Fig. 3

(a) Amino acid → 2-Keto acid → Amino acid → 2-Keto acid → 2-Hydroxy acid dehydrogenase → 2-Hydroxy acid

(b) Table:

| Amino acid   | 2-Keto acid | 2-Hydroxy acid |
|--------------|-------------|----------------|
| Isoleucine   | KRAA        | NADH           |
| Aspartate    | 2-Oxoglutamate | Arginase acid |
| Tyrosine     | HPA         | HPYLA          |
| Tryptophan   | Indolepyruvate | Indolelactate |

(c) Graph showing endogenous metabolites with star symbols indicating significant changes.
Fig. 4
Table 1. The relative susceptibility (IC\textsubscript{50}) of different \textit{L. donovani} strains to PMM. The data shows the means IC\textsubscript{50} from a minimum three separate experiments. *p < 0.05 compared to corresponding WT.

| Strain | Isolate  | PMM IC\textsubscript{50} (µM ± SD) |     |     |
|--------|----------|-----------------------------------|-----|-----|
|        |          | Promastigote                      | Amastigote |    |
| Sb-S   | WT       | 354 ±4                            | 166 ±11    |
|        | PMM-R parent | 116 ± 29                     | 149 ± 20   |
|        | PMM-R clone 8 | 305 ± 27                      | 155 ± 26   |
| Sb-I   | WT       | 56 ± 2                            | 56 ±18     |
|        | PMM-R parent | 455 ± 6*                       | 156 ± 20*  |
|        | PMM-R clone 8 | 481 ± 84*                      | 195 ±12*   |
| Sb-R   | WT       | 85 ± 2                            | 67 ± 1     |
|        | PMM-R parent | 455 ± 6*                       | 165 ± 20*  |
|        | PMM-R clone 9 | 455 ± 6*                      | 165 ± 20*  |

Table 2. The susceptibility of (IC\textsubscript{50}) different \textit{L. donovani} strains to SIN or SNAP treatment. The mean IC\textsubscript{50} values are from 3 separate experiments. *p < 0.05, compared to respective WT.

| Strain | Mean SNAP IC\textsubscript{50} (µM ± SD) | Mean SIN-1 IC\textsubscript{50} (µM ± SD) |
|--------|------------------------------------------|------------------------------------------|
|        | Promastigotes | Amastigotes | Promastigotes | Amastigotes |
| Sb-S   | 117 ± 14 | 111 ± 22 | 1242 ± 62 |
| Sb-S PMM-R clone 8 | 84 ± 16 | 285 ± 38* | 1537 ± 31 |
| Sb-I   | 239 ± 26 | 97 ± 5 | 961 ± 43 |
| Sb-I PMM-R clone 8 | 96 ± 8 | 2456 ± 342* | 306 ± 29* |
| Sb-R   | 57 ± 8 | 934 ± 41 | 313 ± 29 |
| Sb-R PMM-R clone 9 | 45 ± 1* | 1283 ± 132* | 343 ± 37 |