Amphotericin B resistance in *Leishmania mexicana*: Alterations to sterol metabolism, lipid transport and oxidative stress response.

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Abstract

Amphotericin B is increasingly used in treatment of leishmaniasis. Here, fourteen independent lines of *Leishmania mexicana* and one *L. infantum* line were selected for resistance to either amphotericin B or the related polyene antimicrobial, nystatin. Sterol profiling revealed that, in each line, the predominant ergostane-type sterol of wild-type cells was replaced by other sterol species. Broadly, two different profiles emerged among the resistant lines. Whole genome sequencing then showed that these distinct profiles were due either to mutations in the sterol methyl transferase (C24SMT) gene locus or the sterol C5 desaturase (C5DS) gene. In three lines an additional deletion of the miltefosine transporter was found. Differences in sensitivity to amphotericin B were apparent, depending on whether cells were grown in HOMEM, supplemented with foetal bovine serum, or a serum free defined medium (DM). These differences appeared to relate to the presence of lipids in the former. Metabolomic analysis after exposure to AmB showed that a large increase in glucose flux via the pentose phosphate pathway preceded cell death in cells sustained in HOMEM but not DM, indicating the oxidative stress was more significantly induced under HOMEM conditions. Several of the lines were tested for ability to infect macrophages and replicate as amastigote forms, alongside their ability to establish infections in mice. While several lines showed reduced virulence, at least one AmB resistant line displayed heightened virulence in

48 mice whilst retaining its resistance phenotype, emphasising the risks of resistance emerging
49 to this critical drug.

50 **Introduction**

51 The Leishmaniasis are caused by parasitic protozoa of the genus *Leishmania* and are
52 transmitted via the bite of infected female phlebotomine sand flies. Clinical forms range from
53 self-healing but disfiguring cutaneous (CL) and mucocutaneous forms (MCL) (1–3) to
54 potentially fatal visceral leishmaniasis (VL) (2), which can also lead to post-kala-azar dermal
55 leishmaniasis (PKDL) in some instances following treatment (4). No vaccine against human
56 leishmaniasis exists (5), and patient management relies on chemotherapy which is currently
57 unsatisfactory for reasons including cost, toxicity (6,7), drug resistance (1,8–10) and duration
58 and mode of administration. Amphotericin B (AmB) is a natural product used to treat
59 leishmaniasis (11,12). It is a polyene antimicrobial and was initially introduced for anti-
60 fungal use alongside other polyenes, such as nystatin (Nys) (13–21). The introduction of
61 liposomal formulations of AmB, AmBisome®, significantly reduced inherent toxicity (12,22–
62 24) and has become the preferred treatment for VL (8,25,26), especially while costs have
63 been kept low due to donations of the drug to the WHO for use in countries most affected by
64 the disease (27–32). AmB is used in the elimination program of VL in south Asia (1) and has
65 remained effective in VL patients refractory to other antileishmanials (33). It is also active
66 against other forms of the disease (34,35), although it has been used less for CL (12,36). The
67 availability of donated AmBisome® has enabled a campaign of single dose use of the drug.
68 Treating large numbers of individuals in this way avoids complications associated with
69 multiple-dosing regimens and has been of public health benefit (37–39), contributing to a
70 decline in infections as part of a WHO led campaign to eliminate VL as a public health
71 problem (40,41). That campaign, however, has assumed that the development of resistance to

AmB in *Leishmania* parasites is not a significant problem. This is based on the widely held perception that, despite over 70 years of clinical use as an anti-fungal agent, this use has not been accompanied by a significant emergence of AmB resistance (AmBR) in fungal species (42–49). AmB, and other antimicrobial polyenes, bind to ergostane-based membrane sterols with high avidity, and ergosterol and related sterols are the predominant forms in fungi and also in *Leishmania* spp. (50–55). The compounds bind to cholesterol, the major sterol in mammalian cell membranes, with markedly less avidity (56). This selectivity is driven by the altered structural configuration of ergosterol and its analogues relative to cholesterol: differences include double bonds at positions C7 and C22, and a methylation at C24 of the side chain (57–59).

In spite of the perception that AmBR is only a minor risk, several examples of resistance (60–65) and treatment failure with AmB (47,66–68) have been described in leishmaniasis cases, and polyene resistance is by no means unknown in fungal disease (49,64,69–79). An outbreak of autochthonous canine VL in Uruguay found clinical isolates 3 to 4-fold less susceptible to AmB (80,81). Similar differences in susceptibility to AmB were found in a non-endemic region in India (63) and also patients with cutaneous leishmaniasis in Colombia (82).

Development of AmBR is a major threat for the treatment of VL (83). Mechanisms of resistance appear to be diverse. For example, fungal species with a normal content of ergosterol and high levels of catalase are intrinsically resistant to AmB (45,84,85). Furthermore, in *Leishmania* spp., numerous enzymes involved in the protecting against reactive oxygen species (ROS) appear to associate with AmBR. These include the polyamine-trypanothione pathway (PTP) (60,86,87), L-asparaginase (88), cysteine synthase (89), ascorbate peroxidase (90), trypanedoxin peroxidase (91) and the silent information

regulator 2 (Sir2) protein (92). Furthermore, cross resistance to AmB is sometimes observed between lines resistant to the antileishmanials miltefosine and antimonials (13,65,93,94), reflecting a multifactorial mode of action (MoA) of AmB (43,60).

We have previously shown that ergosterol loss relates to selection of AmBR in laboratory-generated mutants of *Leishmania mexicana*. Genome sequence analysis of resistant mutants has shown that mutations in genes encoding lanosterol 14- α demethylase (C14DM) (95), C5-sterol desaturase (C5DS) and C-24 sterol methyl-transferase (C24SMT) (96) can all lead to AmBR. Additionally, sterol profiling revealed loss of ergosterol with inferred loss of drug binding in each case. Other studies in AmBR *Leishmania* spp. have also shown loss of expression of one of the two C24SMT-transcripts (60,97–99) and structural variations at this locus, alongside loss of function of the miltefosine transporter in some instances (93,100–102). Moreover, using *L. major* and *L. donovani*, a series of gene knockout studies have also shown that loss of C14DM (103–105), C24SMT (99,106) and C5DS (107) all lead to the creation of viable and AmBR-parasites, albeit of varying degrees of fitness. Here we describe the characterisation of 15 new independently selected polyene-resistant lines of *L. mexicana* (ten selected to AmB and four to Nys), plus one AmBR-*L. infantum* line. In each case sterol profiling pointed to a loss of ergosterol and its analogue with essentially two different types of compensatory-profile arising, which are each accounted for by mutations to mutations in C24SMT or C5DS. We also assessed the fitness of the selected lines in mice and stability of the resistance phenotype.

Results

Polyene resistance selection in fifteen independent *Leishmania* spp. lines

A total of fifteen independent lines of *Leishmania* spp. (S1 Table) were selected for resistance via a stepwise increasing concentration (S1 Fig) of the polyenes AmB or Nys being

added to the culture media HOMEM or a serum-free defined medium (DM hereon) (S2 Table). Seven AmB resistant *L. mexicana* lines (AmBRcl.14, AmBRcl.8, AmBRcl.6, AmBRcl.3, AmBRA4, AmBRB2 and AmBRC1) were selected in complete HOMEM and three AmB resistant lines (AmBRA3-DM, AmBRB2-DM and AmBRC2-DM) were selected in DM. In addition, four Nys resistant (NysR) *L. mexicana* lines (NysRcl.B2 NysRcl.C1, NysRcl.E1, and NysRcl.F2) and one AmBR *L. infantum* (AmBRcl.G5) were also selected in complete HOMEM. The levels of resistance attained in *L. mexicana* after AmB exposure for 210 days in HOMEM were between 4.5- and 12.8-fold compared with the wild type cultured in parallel in the absence of drug. A comparable increase in resistance was observed in all four lines exposed to Nys for 140 days ranging from 6.6- to 11-fold and to a lesser extent in AmBRcl.G5 (*L. infantum*) which increased by 3.7-fold after drug pressure for 105 days. In contrast, resistance attained in all three AmBR *L. mexicana* lines selected in DM over 372 days was only 2.2-fold that of the wild type in DM. Levels of resistance to AmB or Nys were stable after at least 10 passages in the absence of drug and confirmed in at least two clonal populations derived by limiting dilution from each of the independent fourteen polyene resistant *L. mexicana* lines (for AmBR-*L. infantum*, only one clonal population could be recovered and analysed). AmB can be sequestered by cholesterol and other lipoproteins present in the serum (108,109). Surprisingly, wild type cells sustained in serum-free conditions (DM) were 3.6-fold less susceptible to AmB (EC_{50} 217.3) than they were in HOMEM (EC_{50} 60 nM) indicating an enhanced overall potency of the drug against parasites in rich medium. Parasite growth rate of AmBR and NysR resistant lines cultured in HOMEM was similar to the parental line cultured in parallel without drug whilst in DM AmBR resistant lines was slightly lower (S2A-C Fig). Interestingly, four AmBR lines sustained in HOMEM alongside the parental line showed no growth after switching from HOMEM to DM (S2D Fig). All the resistant cells were morphologically comparable to wild type (WT)

with the exception of AmBRcl.14 and AmBRcl.3 that had an increased cell body length relative to wild type (S2E Fig).

Response of polyene resistant lines to other antileishmanials

Susceptibility to other antileishmanials was measured in only one resistant clonal population derived from each of the fifteen lines by limiting dilution (S1 Table). Considering the similar mode of action between polyenes, we expected reciprocal cross-resistance between AmBR lines to Nys and *vice versa*. AmBR lines were 12.9 to 25-fold cross resistant to Nys. To a lesser extent (1.8 to 2.0-fold), cross-resistance was also apparent with the smaller polyene natamycin (NMC). Similarly, NysR lines cross-resistance to AmB was from 5.8 to 9.1-fold. Variable cross resistance to miltefosine (MF) was found across the polyene resistant lines with the lowest change found in lines cultured in DM (1.1 to 1.4-fold) while cells selected in HOMEM showed changes ranging from 2.3 to 5.3-fold increases in most lines, and, rising to 10-fold in two lines with a deletion of the miltefosine transporter (described in a later section) AmBRcl.8 and AmBRcl.6 (Fig 1). In previous studies, this locus has been associated with cross resistance between AmB and MF (93,100–102). A range of changes in EC_{50} were obtained with two inhibitors of lanosterol 14- α demethylase (C14DM), ketoconazole and fenarimol (103,110–113), and to potassium antimony tartrate (PAT). All fourteen polyene-resistant *L. mexicana* lines cultured in HOMEM and DM were significantly more susceptible to paromomycin (PAR) ($P=0.0001$ to 0.0319), with a decrease of 1.7 to 10-fold in the EC_{50} irrespective of the culture medium (Fig 1, S3 Table and S3 Fig). Similarly, significant hypersusceptibility to pentamidine (PENT) and to methylene blue (MB) was found in all polyene resistant lines.

Fig 1. Fold change in EC₅₀ in all polyene resistant lines of *Leishmania* spp.

Log Fold change (EC₅₀) in resistant lines is relative to the respective parental wild type cultured in parallel without drug. **A-B)** AmBR-*L. mexicana* in HOMEM, **C)** NysR-*L. mexicana* in HOMEM, **D)** AmBR-*L. mexicana* in DM and **E)** AmBR-*L. infantum* in HOMEM. Values higher and lower than 1, indicate a decrease and rise in susceptibility relative to the parental line, respectively. A complete list of values in fold-change and EC₅₀ with statistical analysis is provided in S3 Table and S3 Fig. AmB (amphotericin B), Nys (nystatin), Keto (ketoconazole), MF (miltefosine), PAR (paromomycin), PAT (antimony potassium tartrate), PENT (pentamidine) and MB (methylene blue).

EC₅₀ value to PENT decreased by 7- to 10.9-fold (P=0.0008 to P<0.0001) in all seven AmBR lines and from 2- to 3.8-fold (P=0.01 to P<0.0001) in all four NysR lines, selected in HOMEM (Fig 1, S3 Table and S3 Fig). Recently, Mukherjee et al. related changes in sterol composition to altered mitochondrial membrane potential ($\Delta\psi$ M) which they implicated in

hypersensitivity to PENT whose uptake into mitochondria depends on $\Delta\psi$ M (105). To a lesser extent, AmBR lines grown in DM also revealed hyper-susceptibility to PENT ($P=0.0938$ and $P=0.2143$), except AmBRB2 which was marginally less sensitive (1.15-fold) to the drug. It is possible the differences in oxidative stress induction by AmB in different media also relate to this difference in PENT sensitivity between resistant lines selected in different media. A comparable rise in susceptibility to PENT ($P \leq 0.05$) was also observed in clemastine fumarate-resistant *L. major* promastigotes with altered membrane lipid composition (114), further indicating potential trade-offs between AmB and PENT resistance mechanisms, centred around lipid production. Exposure to MB, an oxidative stress inducing agent (115), showed that AmBR lines grown in HOMEM were 2.9 to 15.3-fold more susceptible than wild type cells ($P=0.0001$ to 0.0084) to this agent which induces intracellular oxidative stress by increasing the NADP⁺ : NADPH ratio and stimulating the oxidation of key cellular redox related thiols such as glutathione or its analogue trypanothione in trypanosomatids (116,117). To a lesser extent (1.76 to 3.34-fold), hyper-susceptibility to MB was also found in AmBR lines selected in DM (AmBRA4, $P=0.506$; AmBRB2, $P=0.114$ and AmBRC1, $P=0.012$) (Fig 1D, S3 Table and S3 Fig). Increased susceptibility to oxidative stress was observed in other AmBR lines exposed to glucose oxidase which creates hydrogen peroxide in medium (95,101).

Metabolic response to AmB exposure

AmB kills *Leishmania* very quickly. At high concentration of AmB (i.e., 5 x the EC_{50}) death ensues within 30 min. We used untargeted metabolomics to determine what changes occur to cellular metabolism over 15 mins by exposing parasites to high concentrations of AmB equivalent to 5x their respective EC_{50} values in HOMEM AmB: 0.31 μ M (parents LmWT1 and LmWT2); 3.0 μ M (AmBRcl.14 and AmBRcl.8) and 1.74 μ M (AmBRB2). Likewise, cell

lines cultured in DM were subjected to AmB at high concentrations (i.e., 5 x EC₅₀): 1.16 μ M (LmWT-DM) and 3.58 μ M (AmBRA3-DM). In untreated cells no significant differences were seen in intracellular metabolites involved in the tricarboxylic acid cycle (TCA) or the PPP between HOMEM and DM (S4 Table).

Fig 2. Metabolic effects of exposure to AmB in *L. mexicana* promastigotes cultured in HOMEM and DM.

Mid log AmBR lines alongside with their parental wild-type *L. mexicana* promastigotes (1×10^8) were cultured in HOMEM (LmWT1, AmBRcl.14, AmBRcl.8, LmWT2 and AmBR2) and DM (LmWT-DM and AmBRA3-DM). **A)** Volcano plot showing significant log₂ fold-changes (red dots) of peaks as detected by LC-MS in promastigotes cultured in HOMEM (AmBRcl.8) and **B)** in DM (AmBRA3-DM). Multivariate data analysis was performed with PiMP analysis pipeline (118) and the Benjamini-Hochberg procedure adjusted raw P-values (q-values) < 0.05 for ANOVA. **C)** Heat map of representative intermediates of the PPP. Each line represents one biological replicate (n=4). Increase (red) or decrease (blue) in fold-change is relative to untreated cells after treatment with AmB at 5 x their EC₅₀ values for 15 mins. Values in bold indicate metabolites identified and all others are putative based on mass. Empty boxes (no value) indicate that the metabolite was not detected. A full list of P-values of the metabolome (including PPP intermediates) is provided in S4 Table.

We then compared the metabolomes of cells exposed to drug that had been cultured under the differing media conditions. The most notable difference was in the major increase in metabolites of the pentose phosphate pathway (PPP): metabolites such as 6-phospho-D-gluconate (Adj. P-value = 0.0121 in AmBRcl.14; 0.0063 in AmBRcl.8; 0.0221 in AmBRB2 and 0.0074 in AmBRA3-DM), glyceraldehyde-3-phosphate (Adj. P-value = 0.1377 in AmBRcl.14; ND in AmBRcl.8; 0.0182 in AmBRB2 and 0.0012 in AmBRA3-DM), sedoheptulose7-phosphate, (Adj. P-value = 0.6936 in AmBRcl.14; 0.0026 in AmBRcl.8; 0.0099 in AmBRB2 and 0.0003 in AmBRA3-DM), ribose 5-phosphate (Adj. P-value = <0.0001 in AmBRcl.14; ND in AmBRcl.8; 0.0016 in AmBRB2 and ND in AmBRA3-DM) and D-glucono-1,5-lactone 6-phosphate (Adj. P-value = 0.0002 in AmBRcl.8; 0.0057 in AmBRB2, not detected in other lines) were significantly increased in abundance in cells exposed to drug in HOMEM (Fig 2A) but not in DM medium (Fig 2B). Upregulation of the PPP (Fig 2C) points to an increased oxidative stress response (119–121). This difference may explain why HOMEM-cultured cells are more sensitive to drug and to the oxidative stress inducer methylene blue (Fig 1 and S3 Table), with these cells experiencing heightened oxidative stress over and above that induced through cellular lysis following pore formation (triggered via AmB-ergosterol binding). This observation may also explain why the fold-change in resistance of the lines selected in HOMEM (5.29 to 12.79-fold) is greater than those achieved in DM (1.36 to 2.54-fold) (Fig 1 and S3 Fig), as alterations in oxidative stress response may greatly enhance HOMEM-based resistance.

Sterol profiling in polyene resistant lines of *Leishmania* spp.

Binding to ergosterol is the primary event in the mode of action of antimicrobial polyenes (43,122). Previous studies with AmBR *Leishmania* spp. have shown that loss of ergosterol commonly accompanies resistance development (60,97,101,123–126). Fig 3 shows the sterol

244 biosynthesis pathway (SBP) in *Leishmania* adapted from the KEGG database
245 (<https://www.genome.jp/kegg/>) and previous work (101,127–129).

246

247 **Fig 3. Sterol Biosynthesis in *Leishmania* spp.**

248 **A)** Numbering system of the carbons in the sterol ring (boxed in yellow) and side chain (boxed in blue). **B)**
249 Molecules coloured indicate sterols found in this work. Enzymes identified in *Leishmania* spp. (solid arrows /
250 boxes) are sterol C14 demethylase, *LmxM.11.1100* (C14DM); sterol C24-methyltransferase, *LmxM.36.2380* and
251 *LmxM.36.2390* (C24SMT) (blue); C8-sterol isomerase, *LmxM.08_29.2140* (C8SI) (green); sterol C5 desaturase
252 or lathosterol oxidase, *LmxM.23.1300* (C5DS) (red); sterol C22 desaturase, *LmxM.29.3550* and/or
253 *LmxM.33.3330* (C22SD); sterol C24 reductase, *LmxM.32.0680* (C24R) and 3- β hydroxysteroid
254 dehydrogenase, *LmxM.18.0080* (3HSD). Also shown some enzymatic steps known in mammals (dotted arrows /
255 boxes). **C)** The position of stigmasta-5,7-dienol within the pathway is unknown. Modified from: KEGG
256 Database (<https://www.genome.jp/kegg/>); Pountain et al, 2019; Yao & Wilson, 2016 (***) and Bansal et al.,
257 2019 (****).

258 The identities and composition of the sterol peaks detected by gas chromatography-mass

259 spectrometry (GC-MS) based on matches with the standards used and with the NIST library

260 are shown in S4 Fig & S5-A Table ([https://www.nist.gov/srd/nist-standard-reference-](https://www.nist.gov/srd/nist-standard-reference-database-1a-v14)

261 [database-1a-v14](https://www.nist.gov/srd/nist-standard-reference-database-1a-v14)). Ergosta-5,7,24-trienol ($C_{28}H_{44}O$) was the most abundant sterol detected

262 here in wild type *Leishmania* spp. promastigotes ranging from 72 to 82% of total sterol (Fig

263 4). Also known as 5-dehydroepisterol, this isomer of ergosterol differs from the major fungal

ergosterol, ergosta-5,7,22-trienol (128) in the position of the double bond (carbon 24 rather than carbon 22) and in its chromatographic retention time (S5-A Table) (103,130).

Fig 4. Percentage of sterols (GC-MS) in polyene resistant lines of *Leishmania* spp.

Standard deviation of the mean (bars) is from three biological replicates. Parasites were cultured as promastigotes in HOMEM (panels A, C and D) and DM (panel B) or as axenic amastigotes (recovered after infection in mice) in Schneider's media (panel E). Species are *L. mexicana* (panels A, B, D and E) and *L. infantum* (panel C) and lines are resistant to polyenes AmB (panels A, B, C and E) and Nys (panel D). See S5 Table and Material and Methods for a full description of the content and identification of sterols with GC-MS.

Another abundant sterol in wild type promastigotes was ergosta-7,22-dienol (11%) followed by cholesterol (2 to 7%) (Fig 4). Cholesterol was absent in all AmBR and wild type parasites cultured in DM (Fig 4D), which is unsurprising given that cholesterol is derived from exogenous sources, including the serum added to culture media (131,132) and from the membrane of the host-macrophage (133). This possibly explains its higher abundance in the intracellular stage of the parasite in both wild type (12.2%) and AmBR lines (7.3 to 10.2%) cultured in HOMEM (Fig 4E & S5-B Table). All of the polyene-selected lines had altered sterol profiles compared to wild-type and these fell into two broad types. In three of the AmBR *L. mexicana* lines selected in HOMEM and all four of the Nys resistant *L. mexicana* lines, the predominant sterol became ergosta-7,22-dienol (96 to 97%) (Fig 4A & D), which

lacks the C5 desaturation of AmB-binding ergosterol. Another sterol, stigmasta-5,7-dienol, was also present in all NysR lines, more abundant in NysRcl.B2 (9.9%), NysRcl.C1 (14.2%), NysRcl.E1 (6.1%) and NysRcl.F2 (7.0%).

We also analysed the sterol content of axenic amastigotes in two resistant lines alongside with their parental wild type following recovery from mouse tissue (post-infection) parasites were maintained as promastigotes in HOMEM and transformed into amastigotes in Schneider's medium. Interestingly, stigmasta-5,7-dienol was also present in wild type *L. mexicana* axenic amastigotes (8.1% of total) and unchanged or little depleted in AmBRcl.8 (5.8%) while increased notably in AmBRcl.14 (49.1%) (Fig 4E). However, its role in resistance and its position within the sterol biosynthetic pathway awaits further investigation (Fig 3C). In five of the HOMEM selected *L. mexicana* AmB lines and all of the AmB lines selected in DM, as well as the *L. infantum*, cholesta-5,7,22 trienol became predominant. In cells selected in HOMEM it comprised 86%-93% of the total sterol, while in those cells selected in DM this proportion was somewhat lower (69.7%-75.4%) whilst an isomer, cholesta-5,7,24-trienol (C₂₇H₄₂O) increased from 3.4% abundance in wild-type to 7.9%-12.3% in resistant lines selected in DM. In AmBR *L. infantum* 64% of the total comprised this sterol while a further 25% of the total was its isomer cholesta-5,7,24 trienol. Interestingly, cholesta-5,7,22-trienol was higher in wild type cells sustained in DM (16.5%) in comparison with values found in all parental lines cultured in HOMEM (0.5 to 2.57%) (Fig 4A-C and S5-B Table).

Genomic changes identified with whole genome sequencing in polyene-resistant *Leishmania* spp. lines

Whole genome sequencing was performed on each of the 15 polyene resistant cell lines and particular attention paid to gene deletion or amplification and SNPs found repeatedly in the

same gene in different isolates. Given the altered sterol profiles reported in the previous section, we also systematically inspected all genes encoding enzymes of the sterol pathway. All polyene resistant *Leishmania* lines analysed here revealed mutations to one or other of two genes, sterol C5-desaturase (C5DS) and sterol C24-methyl transferase (C24SMT), encoding enzymes of the sterol pathway.

Sterol C5 desaturase mutations

One group of three resistant lines revealed point mutations to C5DS (or lathosterol oxidase (LOX), gene ID = *LmxM.23.1300*). In AmBcl.3, all of the reads of C5DS are mutated (S4 Fig) containing one of two independent heterozygous mutations within the same codon, G220A (V74M) and T221A (V74E), leading to a substitution of a non-polar valine residue, which is conserved across the aligned *Leishmania* species (S5 Fig), by a polar methionine and a negatively charged glutamate residue, respectively. A second homozygous substitution G731T (R244L) was present in AmBRcl.3 and AmBRcl.14 (Fig 5A). Interestingly, both lines also showed a homozygous variant G208A (G70S) that changed a non-polar glycine into a polar serine residue in the adjacent gene downstream from C5DS, a dynein heavy chain (ID = *LmxM.23.1310*) (Fig 5B). Also, in C5DS, AmBRcl.14 showed an in-frame deletion ATG277-279 (M93del) while in NysRcl.B2 a neighbouring frame-shift deletion G286del (A96del) was present (Fig 5A-B), leading to loss of a full-length coding region in each case. In all these C5DS mutants an accumulation of ergosta-7,22-dienol (Fig 4) was observed, indicating that all of these different mutations impacted upon activity of the enzyme leading to an accumulation of its substrate whose use is ablated in the mutants.

Fig 5. Mutations in lipid-associated genes found in polyene resistant lines of *L. mexicana*.

A) Coverage is shown in colours for NysR (top in pink), AmBRcl.14 (upper-middle in yellow), AmBRcl.3 (lower middle in orange) and wild type (WT) (bottom in green) and reads (grey bars) with mapping quality, > 0 are shown for each line. The blue line at the bottom indicates the open reading frame of the C5DS (or LOX) *LmxM.23.1300* gene and variants are indicated (empty arrows). Image produced with WGS data aligned to the reference genome (<https://tritrypdb.org>) using IGV_2.8.9 (<http://software.broadinstitute.org>); **B)** Cartoon showing the variants linked to polyene resistance in LOX (C5DS) (blue bar), a SNP in the gene downstream (*LmxM.23.1310*) is also shown (red bar). The red circles indicate that these two mutations are in the same codon and are independent (found in different reads). Mutations found in AmBRcl.14 and AmBRcl.3 (in black) and in Nyscl.B2 (blue) are shown alongside with a variant G139R (red) reported in our study (Pountain et al, 2019) which indicates a residue involved in catalysis or ligand binding; **C)** Cartoon of MT gene *LmxM.13.1530* portraying conserved domains and the proximity of the variant W347* (stop codon) found in this study in AmBRC1 (green circle).

Sterol C24 methyl transferase mutants

The second sterol pathway gene that was mutated in multiple separate lines was the C24SMT tandem array (*LmxM.36.2380* and *LmxM.36.2390* in *L. mexicana*) (S6 Fig & Table 1). Five AmBR lines of *L. mexicana* cultured in HOMEM, AmBRcl.8, AmBRcl.6, AmBRA4, AmBRB2 and AmBRC1 showed no coverage of the intergenic region (~2.5 kb) between the two copies (S6A-B Fig). The *L. infantum* AmB resistant line (AmBRcl.G5) also showed a loss of coverage across this intergenic region (S6E Fig). Copy number variation (CNV)

confirmed a deletion within the C24SMT tandem pair in all of these lines (S8 Table). In AmBRA4, a novel non-silent mutation A325V (C974T) resulted in a substitution from alanine to valine. In all of the AmB resistant lines selected in DM, this intergenic region was not of decreased coverage (S6C Fig), but a variant G961A (V321I) which constitutes a locus that differs between the two copies of C24SMT (Pountain et al, 2019) was found. All of the lines where mutations were targeted around the C24SMT locus demonstrated an increase in cholesta-5,7,22-trienol (Fig 4A-C), which is the substrate for C24SMT, indicating that in each case the mutations have diminished the activity of that enzyme. This locus showed no genomic changes in NysRcl.B2 (S6D Fig), in which the predominant sterol was ergosta-7,22-dienol (Fig 4 and S5-B Table) compatible with a mutation in C5DS as noted above.

Mutations to the Miltefosine transporter and genes involved in oxidative stress defence

Several previous studies on resistance to AmB (93,101) showed that the miltefosine transporter gene (*LmxM.13.1530*) was also deleted during the process of selection of AmB resistance. Here we noted that in three lines (AmBRcl.8, AmBRcl.6 and AmBRA4) the MT and an adjacent gene (*LmxM.13.1540*) were deleted (S7C-D Fig). In AmBRA4 the deleted region was larger (~20 kb) spanning two additional genes (S7D Fig). CNV analysis confirmed that the MT gene was, however, retained in all of the other lines. A reduction in CNV in this locus was also found in AmBRcl.14, AmBRcl.3 and NysRcl.B2 (they lost one copy of the two) but increased in AmBRB2. Changes in ploidy in all lines are shown in S8 Fig and S8 Table). In all of the lines selected in DM, the MT gene remains (S7E Fig), but in one line, AmBRC1, a homozygous variant G1041A (W347*) appears, indicating a translation termination (stop codon) and consequent loss of function of MT that resulted in 3.3-fold resistance to MF relative to WT (Fig 5C & Table 1).

373

374 **Table 1. WGS in sterol and non-sterol genes in polyene resistant lines of *Leishmania* spp.**

375 Gene IDs were retrieved from TriTrypDB in *L. mexicana*. Genotype and amino acid changes from variants in
 376 sterol genes is provided in S6 Table. A complete list of variants is provided for SNPs (S9 Table) and CNVs (S8
 377 Table). Hom: homozygous; Het: Heterozygous.

Gene ID and name	Mutation locus	Resistant line(s) with the mutation	Sterol intermediate accumulated	Culture media
Sterol genes				
<i>LmxM.23.1300</i> C-5 desaturase (C5DS)	1. G220A (V74M)	AmBRcl.3 (SNPs 1 and 2 are independent)	ergosta-7,22-dienol	HOMEM
	2. T221A (V74E)			
	3. G731T (R244L)	AmBRcl.3 and AmBRcl.14		
	4. ATG277-279 (M93del)	AmBRcl.14		
	5. G286del (A96del)	NysRcl.B2		
<i>LmxM.36.2380</i> / <i>LmxM.36.2390</i> C-24 sterol methyl transferase (C24SMT)	6. G961A (V321I)	Hom: AmBRcl.3, AmBRcl.8 and AmBRcl.6; AmBRA3-DM, AmBRA4 and LiWT	cholesta 5,7,22	HOMEM
		Het: LmWT1, AmBRcl.14, LmWT2, AmBRB2, AmBRC1, LmWT3, NysRcl.B2.		DM
		Hom: AmBRB2-DM, AmBRC2-DM Het: LmWT-DM		
	7. Intergenic region with partial or total absence of coverage and decrease in CNV	<i>L. mexicana</i> : AmBRcl.8, AmBRcl.6, AmBRA4, AmBRB2 and AmBRC1 (S6A-B Fig). <i>L. infantum</i> : AmBRcl.G5, LiWT (S6E Fig)		HOMEM
	8. C974T (A325V)	AmBRA4 (in <i>LmxM.36.2390</i>)		
	Non-sterol genes			
<i>LmxM.13.1530</i> Miltefosine transporter and adjacent genes <i>LmxM.13.1540</i> (upstream), and <i>LmxM.13.1510</i> , <i>LmxM.13.1520</i> (downstream)	9. Deletion of a ~9 to 26 kb region spanning various open reading frames (ORF) and a decreased in CNV	AmBRcl.8, AmBRcl.6 and AmBRA4 (S7 Fig).	cholesta 5,7,22	HOMEM
	10. G1041A (W347*)	Hom: nonsense mutation (stop gained) in AmBRC1		
<i>LmxM.25.2380</i> Glutathionyl-spermidine synthase (GSS) (LINF_250031200 in <i>L. infantum</i>)	11. G575C (S192T)	Het: variant in AmBRcl.8 and AmBRcl.6 (<i>L. mexicana</i>) and AmBRcl.G5 (<i>L. infantum</i>) (S9 Fig)	cholesta 5,7,22	HOMEM

Other genes beyond those encoding members of the sterol pathway were also found in two or more polyene-resistant lines and these are listed in Table 1, S10 Fig and S9 Table and their roles or actual relationship with resistance awaits further analysis, although it is notable that four genes encoding enzymes associated with the leishmania thiol redox system (S9B Fig) were found. Notably, these mutations are present in cells selected in HOMEM medium in which oxidative stress was more profound. In AmBRcl.G5 (*L. infantum*), a heterozygous non-synonymous mutation G575C (S192T) (S9A Fig) was present in the glutathionylspermidine synthase (GSS) encoding gene (ID = *LINF_250031200*, formerly annotated as *LinJ25.2500*). Strikingly, the same residue G575C (S192T) in GSS (*LmxM.25.2380*) was also mutated in two *L. mexicana* lines, AmBRcl.8 and AmBRcl.6 (S9C Fig) both which showed an attenuated phenotype in mice (Fig 6-A). Moreover, in five lines, CNV revealed relatively small changes in another four genes involved in oxidative stress, two copies of the type II trypanothione peroxidase (glutathione peroxidase-like) (*LmxM.26.0800* and *LmxM.26.0810*), glutaredoxin (*LmxM.26.1540*) and glutathione S-transferase (*LmxM.12.0360*). Previous work has pointed to a potential role of the leishmanial redox system (S9B Fig) in resistance to AmB (60,87–89,91,92,134). The actual role of these genes awaits further investigation, but it seems likely that changes to the redox system might indeed be important particularly during selection in HOMEM where higher levels of oxidative stress in cells exposed to AmB are manifest.

Infectivity of AmBR lines *in vivo*

We initially selected AmB resistance in cultured promastigotes given the tractability of this system for biochemical analysis. However, it is important to understand whether findings from promastigotes translate into amastigote forms that replicate in mammalian macrophages and which are associated with disease. Therefore, we chose four of the AmBR mutants to test

their ability to retain infectivity and virulence in mice. Two of these were from the C5DS mutant group (AmBRcl.14 and AmBRcl.3) and two from the C24SMT mutant group (AmBRcl.8 and AmBRcl.6), in these groups, the wild type sterol (ergosterol) was replaced by ergosta-7,22-dienol and cholesta-5,7,22-trienol, respectively. For these experiments, we also included a low passage (P2) isolate (AmBRcl.14 PI) recovered from a previous infection with AmBRcl.14. Notably, mice infected with AmBRcl.14 (C5DS), AmBRcl.3 (C5DS) and AmBRcl.14 PI (C5DS) showed an increase in the size of the lesion at the inoculation site (footpad) up to two times greater (week thirteen) than that observed in mice infected with the parental wild type (Fig 6-A). By contrast, mice infected with AmBRcl.8 (C24SMT) and AmBRcl.6 (C24SMT) showed no macroscopic alterations along the entire experiment and no parasites from these two lines were identified using histology suggesting a fitness loss in these two lines (S11 Fig). Interestingly, material excised from footpad injection sites and associated lymph nodes from all animals was placed into HOMEM yielding proliferative AmBR promastigote cultures from all four AmBR lines and wild type. The AmBRcl.14, AmBRcl.14 PI, AmBRcl.3 and wild type lines reached a density of $\sim 1 \times 10^5$ cells per mL within 72 hours while AmBRcl.8 and AmBRcl.6 required seven days to reach this density. While the lower parasite density obtained with AmBRcl.8 and AmBRcl.6 may be explained by the absence of lesion growth observed in mice, the presence of viable parasites recovered from mice tissue with these two lines, which moreover retained their resistant phenotype was unexpected. Previous work with *L. infantum* showed infectious parasites with a unique sterol profiling (129,130,135). Here, it appears that accumulation of ergostanes (96.0 to 96.7%) due to loss of sterol C5 desaturase (C5DS or LOX) is related to the hyper-infectivity observed in AmBRcl.14 and AmBRcl.3. Conversely the accumulation of cholestane type sterols through loss of C24SMT (73.3 to 87.9%) in AmBRcl.8 and AmBcl.6 (Fig 4 & S5-B Table) may also explain the attenuated phenotype observed in these two mutants. Notably, a unique missense

heterozygous mutation in both attenuated lines AmBRcl.8 and AmBRcl.6 (S9C Fig) and in AmBRcl.G5 of *L. infantum* (S9A Fig) but not in AmBRcl.14 and AmBRcl.3, was present in the glutathionylspermidine synthase (GSS) gene and in three other genes with unknown function (S10 Fig). GSS is part of the leishmanial multi-protein system (S9B Fig) that protects the parasite against redox species and has been previously linked to virulence in related species (136), however, the impact of these heterozygous changes is not known and other virulence factors cannot be ruled out.

Fig 6. AmB activity in polyene resistant *L. mexicana*.

A) Stationary promastigotes were inoculated at 2×10^6 into 500 μ l of PBS. Evolution of lesion was measured weekly. BALB/c female mice were two-months old at the time of inoculation. Treatment: 1 mg per kg IV every other day in the tail vein with a total of six injections ($\sim 120 \mu$ l) of AmB (deoxycholate). **B)** activity of AmB in axenic promastigotes before and after infection of mice; **C)** AmB activity against AmBR- and NysR-L.

mexicana in infected macrophages. Values (μM) are the mean $\text{EC}_{50} \pm \text{SD}$ (bars) (See S7 Table). Tukey's multiple comparison test was used to find pairwise differences between resistant lines and parental wild type. Statistically significant values ($P < 0.05$, 95% confidence interval) are indicated with stars as follows: $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

Retention of resistance *in vivo* and post infection *in vitro* and in macrophages

We also tested whether the parasites from the four lines chosen for *in vivo* infection retained resistance to AmB within the mouse (Fig 6A) and also when recovered from primary lesions (footpad) (Fig 6B & S7 Table) and lymph nodes. After infection, primary lesion size was monitored weekly thereafter and mice were treated with AmB (1 mg per kg) at week eight of infection. In wild type parasites, a plateau in lesion size was attained by fourteen days post-treatment and sustained until the end of the experiment (thirteen weeks). By contrast, mice infected with AmBRcl.14, AmBRcl.3 and AmBRcl.14 PI, the latter recovered from a previous infection, lesion size continued to increase following AmB treatment indicating that these resistant lines were unaffected by this dose of AmB. Due to the absence of increase of lesion in mice infected with AmBRcl.8 and AmBRcl.6 (Fig 6A), retention of resistance in these two lines was assessed *in vitro* as outlined below. Higher dosing was precluded due to the toxicity of the drug, but the data clearly point to all these AmBR parasites being of reduced sensitivity to AmB.

To confirm whether parasites retained resistance after infection, promastigote cultures of the four AmBR lines recovered from footpads and lymph node tissues of infected mice treated with AmB were established and EC_{50} values to AmB measured (S7 Table and S12 Fig). Before infection, AmBR isolates were respectively 11.2-fold (AmBRcl.14, $P = 0.0138$), 12.3-fold (AmBRcl.3, $P = 0.0053$), 12.1-fold (AmBRcl.8, $P = 0.0062$) and 12.8-fold (AmBRcl.6, $P = 0.0036$) less sensitive to AmB than wild type. Comparable resistance was observed in promastigotes recovered post-infection from all AmBR lines and sub-cultured for two passages (P2) *in vitro* with values of 6.84-fold (AmBRcl.14P2, $P = 0.3229$), 10.8-fold

(AmBRcl.3P2, $P=0.0131$), 9.34-fold (AmBRcl.8P2, $P=0.0479$) and 12.3-fold (AmBRcl.6P2, $P=0.0093$) (Fig 6B). We also tested these four lines that had been recovered from mice (plus the Nys resistant NysRcl.B2 from axenic culture) within infected macrophages and confirmed their resistance phenotype. Interestingly, all lines with mutations in C5DS (and accumulation of ergosta-7,22-dienol) were notably less susceptible (11.20-fold in AmBRcl.14P2, $P=0.0005$; 12.2-fold in AmBRcl.3P2, $P=0.0003$ and 13.84-fold in Nyscl.B2, $P=0.0001$) with respect to their parental wild type (Fig 6C and S7 Table), than either of the two attenuated lines (6.52-fold in AmBRcl.8P2, $P=0.0130$ and 4.37-fold in AmBRcl.6P2, $P=0.2100$) which had mutations in the C24SMT gene locus (alongside with a deletion of the miltefosine transporter) in which the accumulation of cholesta-5,7,22 was predominant (Fig 4A). Overall, these findings indicate that the retention of the resistant phenotype was stable across all polyene-resistant lines after being recovered from mice (footpads and lymph nodes) (S12 Fig) irrespective of their genomic variants in sterol genes or their virulence.

Discussion

AmB has emerged as a leading drug to treat leishmaniasis, particularly in its liposomal formulation AmBisome® (8,25,26). Resistance to the drug would impact heavily on efforts to eliminate VL (40,41). For leishmaniasis, several instances of AmB treatment failure have been reported in the field, involving cases not known to involve immunosuppression by HIV (60,68) as well as those in HIV infected immunosuppressed patients (47,66,67,137,138). Moreover, numerous studies have now revealed that resistance can be selected in the laboratory, where the resistance phenotype has frequently been linked to loss of AmB-binding ergostane-type sterols, associated with mutations in enzymes of the sterol pathway. Most often the mutations and loss of expression have arisen in the C24SMT gene locus (60,97,99,139), but in other cases C5DS has been implicated (101) and in one case a loss of

C14DM (CYP51) was identified (95). In each case, the mutations lead to loss of ergosterol and an accumulation of other sterol precursors within the pathway, leading to the conclusion that changes in membrane sterols prevent AmB binding to the parasite membrane with the same avidity as to WT cells thus leading to loss of sensitivity. Such mutations have been discovered in laboratory-generated AmB-resistant parasites, as well as clinical isolates of *L. mexicana* (95,101), *L. donovani* (60,97–99) and *L. infantum* (93).

Apart from our work in which we analysed four AmBR lines (101), previous studies have focused on individual single mutants restricting which restricts the ability to identify different causes of resistance. For this reason, here, we selected a total of 15 new mutants resistant to polyene antimicrobials including 10 *L. mexicana* lines resistant to AmB (AmBR) (7 in HOMEM medium containing FBS and 3 in a serum and lipid free DM). A further four *L. mexicana* lines were selected in HOMEM for resistance to Nys (NysR), a relative of AmB and a single *L. infantum* line was selected for AmBR in HOMEM. Analysis of their respective sterol complements indicated two different profiles had been produced. In both cases, the primary membrane sterol in *Leishmania* spp., ergosterol, was lost and replaced by either ergosta-7, 22-dienol in two of the AmBR *L. mexicana* lines (and in all four NysR lines) selected in HOMEM or cholesta-5,7,22-trienol in five of the HOMEM selected *L. mexicana* AmBR lines and all three of the AmBR lines selected in DM (AmBR-DM), as well as the *L. infantum* AmBR line. In the former group, mutations to C5DS were identified in sequencing the genome while in the latter group mutations to the C24SMT locus were associated. These observations are consistent with previous studies where C24SMT (60,97–99,101) and C5DS (101) have been found associated with development of AmBR. Moreover, experiments in *L. major* where C24SMT (106) and C5DS (107) genes were knocked out of WT parasites also showed that viable parasites with resistance to AmB could be recovered.

Since all of this laboratory-based work has focused on promastigotes forms of the parasites, questions as to their relevance *in vivo* have been made. Purkait et al. isolated parasites from a VL patient who failed treatment and accumulation of sterols indicated changes to C24SMT similar to those found by others in mutants generated under laboratory conditions (97–99,101). This would indicate the potential of C24SMT mutants to propagate as AmBR cells in patients. *L. major* C24SMT-KO mutants derived by Mukherjee et al. (106) were also of reduced virulence in mice, as were the two lines AmBRcl.8 and AmBRcl.6 with C24SMT mutations we tested here. However, Pountain et al. earlier found a C24SMT mutant that retained virulence in mice. This indicates that it might be mutations other than those to C24SMT *per se* that are responsible for attenuation in mice. Here, for example, we identify changes to the glutathionylspermidine synthase (GSS) gene in both attenuated *L. mexicana* lines (AmBRcl.8 and AmBRcl.6) and in *L. infantum* (AmBRcl.G5) (S9A Fig), although do not yet know whether these changes are associated with attenuation. On the other hand, two separate lines (AmBRcl.14 and AmBRcl.3) with variants in C5DS, both retained virulence and resistance to AmB in mice. In *L. major* C5DS-KO lines displayed only a small loss of virulence in mice (99,106,140), thus C5DS mutations can also be selected, yielding parasites both AmBR and still able to infect mammals. This renders the risk of selection of AmBR in *Leishmania* a genuine risk.

Most studies in AmBR fungi, particularly yeasts including *S. cerevisiae* and *Candida* spp. also showed a tendency for C24SMT and C5DS (ERG6 and ERG3 in fungi, respectively) (75,77) mutations although changes in other genes of the sterol pathway (ERG- in fungi) such as C14DM (ERG11) (73,77,141,142), C22-sterol desaturase (ERG5) (73,75) and C-8 sterol isomerase (ERG2) (72,77) are also involved. In yeast, enhanced oxidative stress resistance has also been implicated in loss of susceptibility to AmB (45,84,85). In *Leishmania*, other studies also pointed to oxidative stress response in AmBR (60,87,91,92,119,134,143). Here

we show that the activity of AmB appears to be substantially augmented in rich medium (HOMEM) and associated with enhanced induction of oxidative stress. The activity of AmB against wild type *L. mexicana* grown in HOMEM containing FBS compared with serum-free DM showed the drug to be significantly more potent (3.6 to 4.11-fold) in the rich conditions (Fig 3A,B & D; S3 Table). Metabolomics analysis revealed a significant increase in flux of glucose via the PPP in the cells in rich growth conditions when treated with high concentrations of AmB (Fig 2), which also indicates enhanced oxidative stress causing this increased influx to regenerate NADPH lost in fuelling the redox defence pathways (119–121). Notably, no parasites selected for AmB resistance in DM revealed changes in redox pathway genes, emphasising that environment-specific impacts play key roles in AmB mode of action and resistance.

Overall, this work demonstrates that AmB resistance can be selected relatively easily in *Leishmania* and that changes to sterol profiles predominate. Moreover, we demonstrate that genomic sequencing of multiple lines selected for resistance in parallel can reveal changes that are not identified by studies using single mutants or in which a single gene is knocked out of wild type parasites. As the drug induces oxidative stresses in a manner that is conditional upon the medium in which the parasites grow, then changes to the oxidative stress defence systems can also be important in development of some degree of resistance. When resistance is selected in incremental steps, as done here, a series of events might be behind resistance. In addition to some enzymes involved in the thiol redox systems, three of the lines selected here, as is the case for two earlier demonstrations (101,102) had lost the miltefosine transporter, which led to cross-resistance to the oral antileishmanial MT in these lines not evident in the other lines. Again, it would seem that the incremental stepwise approach to resistance selection might see some cells changing sensitivity to AmB by other membrane changes associated with loss of the miltefosine transporter, which is a membrane

flippase, involved in the architecture of the plasma membrane lipids (93,144–146). The combined roles of redox changes and sterol changes could influence the ability to select and sustain resistance in vivo. For example, two C24SMT mutants were attenuated in mice but an additional mutation in the oxidative defence gene GSS was also found along with a deletion of the MT in each. Further study is required to understand the relative contributions of each. On the other hand, both C5DS mutant lines retained their infectivity in mice. In these, two different independent mutations in the same residue (V74M) and two deletions (M93del and A96del) were found. Whether or not showing virulence, all lines tested in mice retained a stable AmBR phenotype irrespective of their genomic variants in sterol genes or sterol profile.

The fact that AmBR has been rare in the field until now might simply be due to the fact that, until recently, it was not widely used, and generally involved protracted dosing regimens. Extended use and the decision to use a single dose of the drug in monotherapy has great public health benefits (40,41,147,148) but that policy must take into account the fact that selection of resistance is clearly feasible and that resistance can be sustained in parasites that are virulent in mammals. Notably, we observed that, in most cases, AmB resistance is associated with cross-resistance to MT, in some instances, this derived from profound genomic changes such as SNPs and CNVs, as we showed here in three AmBR lines (S7 Fig). Interestingly, here, as in many other studies, AmB resistance is accompanied by increased sensitivity to the antileishmanials PENT and PAR (and to MB). Data from *L. major* evincing a rise in susceptibility to the former were associated with alterations in other membrane lipids (114), which is consistent with interlinked lipid biosynthetic pathways being affected. In the case of PENT, it would seem to be changes in mitochondrial membrane potential due to altered sterol profiles that is responsible (105) and if sterol changes are common then it

would be worth considering use of PENT against AmBR lines should they emerge in the field.

Material and Methods

Cell culture and polyene-resistant selection

L. mexicana (MNYC/BZ/62/M379) and *L. infantum* JPCM5 (MCAN/ES/98/LLM-877) promastigotes were cultured at 25°C and 27°C, respectively, in HOMEM (GE Healthcare and Gibco®) supplemented with 10% v/v heat-inactivated foetal bovine serum (HI-FBS) (Labtech International) or in serum-free defined medium (DM) (149,150). HOMEM and DM culture media (S2 Table) were supplemented with 100 µg per mL streptomycin, and 100 IU mL⁻¹ penicillin. Parasites were sub-cultured weekly starting at a density of 1 x 10⁵ cells per mL and growth rate, cell density and morphology were determined by light microscopy with a haemocytometer (151,152). S1 Table shows all the fifteen-individual polyene-resistant lines selected *in vitro* alongside their respective parental wild type, cultured in parallel in the absence of drug. *L. mexicana* cells were selected for resistance by stepwise increasing concentrations of AmB (ten lines) in HOMEM (thirty-two weeks) starting from 50 nM and rising up to 220-300 nM (S1 Fig-A and C) and in DM (fifty weeks) from 100 nM up to 300 nM (S1 Fig-D). Likewise, AmB (one line) for *L. infantum* in HOMEM (fifteen weeks) started at 50 nM up to 100 nM (S1 Fig-E). Nystatin was added to *L. mexicana* (four lines) in HOMEM from 1.5- up to 12.5 µM (S1 Fig-C). Individual clones from each independent resistant line were obtained by limiting dilution assay (LDA). The most resistant clones (n=15) (S1 Table) were selected for further analyses. Retention of resistance (as promastigotes) was confirmed after at least ten passages without drug pressure and post-infection *in vivo* in all resistant lines (n=5) and wild type included in these experiments. Cell lines were preserved in culture medium with 15% DMSO (v/v). Axenic amastigotes were

cultured at 32.5 °C with 5% CO₂ in Schneider's medium (SDM) pH 5.5, supplemented with HI-FBS (10% v/v), 1.5 mL of hemin (2.5 mg per mL in 50 mM NaOH, 0.003% v/v), 100 µg per mL streptomycin, and 100 IU mL⁻¹ penicillin as described before (153).

Drug susceptibility assays

Drug susceptibility to various drugs was determined using the Alamar Blue assay (154–156) with some modifications. Briefly, mid-log phase promastigotes (1 x 10⁶ cells per mL) were incubated for 72 hours with different concentrations of drugs serially diluted in a two-fold stepwise fashion in 96-well plates, followed by the addition of resazurin (0.49 mM in 1x phosphate-buffered saline (PBS), pH 7.4) and further incubation for 48 hours. For the experiments of retention of resistance within macrophages, incubation with AmB (AmBisome®) and resazurin was for 48 and 6 hours, respectively. Absorbance (fluorescence) was measured using a BMG LabTech Fluostar Optima fluorometer. Intensity was read at λ_{EX} 530 nm and λ_{EM} 590 nm and analysed with Prism 8.0 software to obtain the 50% inhibitory concentration (EC₅₀ ± SD, 95% confidence interval) using regression analysis. One-way ANOVA and Tukey's multiple pairwise comparison tests were performed independently for each compound. All assays were performed in three biological replicates. Unless stated otherwise, all drugs used in these assays were bought from Sigma-Aldrich.

Infection of mice, macrophages and ethics statement

Eight-week-old female BALB/c mice (from Harlan UK Ltd) were kept at the Central Research Facilities of the University of Glasgow, Glasgow U.K and randomly assigned to either of the treatment groups (wild type versus AmBR lines). Infection of mice was performed by inoculating 2 x 10⁶ stationary phase *L. mexicana* promastigotes resuspended in 100-200 µl of filter sterilised PBS in the left footpad. Mice were treated with six doses of

intravenously injected of AmB (1 mg per kg) into the tail vein q.o.d. (every other day) starting at week 8 of infection as described elsewhere (123). Lesion size was assessed weekly before and after chemotherapy (thirteen weeks in total). Amastigotes were recovered post-infection from footpad- and lymph nodes tissue and sub-cultured weekly in Schneider's medium (SDM) as previously described (153), or transformed into promastigotes and maintained in complete HOMEM. Footpad- and lymph nodes samples were processed for histology and stained with haematoxylin and eosin (H&E) at the Beatson Institute, University of Glasgow, and analysed with light microscopy. Mice were maintained and euthanized following the United Kingdom regulations and the Animals (Scientific Procedures) Act, 1986 (ASPA) under licence PCF371688.

Retention of resistance to AmB post infection in mice

Retention of resistance to AmB after infection in mice (NysRcl.B2 was from axenic promastigotes culture) was assessed in both axenic promastigotes and in infected macrophages. In the first case, a low passage (P2) of axenic parasites was recovered from mice tissue (as amastigotes) and transformed into promastigotes in HOMEM and drug susceptibility was performed as with previous assays. Retention of AmB resistance within infected macrophages was performed following a method described elsewhere with some modifications (157–159). Briefly, a macrophage-like cell line (RAW 264.7) derived from BALBc/mice was maintained at 37 °C in a humidified atmosphere with 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, +L-glutamine and 110 mg/L Sodium Pyruvate (Gibco), supplemented with 10% HI-FBS, along with 100 IU and 100 µg/ml each of penicillin and streptomycin. Stationary phase promastigotes (2.5 x 10⁶ per mL) were used to infect adherent macrophage-like cells (2.5 x 10⁵ per mL) at a ratio 10:1 for 24 hours. Infected cells were then washed (3 x) with DMEM to remove extracellular parasites

before the addition of variable concentrations of AmB and further incubation for 48 hours. DMEM was then removed and macrophages were lysed using sodium dodecyl sulfate 0.05% (20 µl per well) for 1 min followed by resuspension in complete HOMEM (180 µl per well) and plates were incubated at 26 °C for 48 hours. The presence of promastigotes was assessed with direct microscopy prior to determination of the EC₅₀ as described before for drug assays.

Sterol analysis by GC-MS

GC-MS was performed at Glasgow Polyomics. Sterols were extracted from parasite pellets for Gas chromatography-mass spectrometry (GC-MS) analysis. Mid-log (3×10^8) axenic promastigotes and amastigotes (after transformation in SDM medium as described previously) (153,160) were washed in PBS and resuspended in 500 µl of KOH-ethanol (20 ml dH₂O in 30 ml EtOH, 12.5 g KOH) and incubated at 85°C for 1 hour in Pyrex glass tubes. A similar volume of n-heptane was added, and samples were vortexed for 30 seconds. After 20 min the organic layer containing the sterols was transferred into glass borosilicate vials with a Teflon cap (Thermo Fisher®) and stored at -80°C until GC-MS analysis. All samples including blanks (no cells) and pooled samples (a mix with 20 µl from each sample) were in triplicate. Sterols were detected as ester derivatives with trimethyl silane (TMS) and were reported as their underivatized form after normalisation. Briefly, samples were dried with N₂ flow at 60 °C in glass vials followed by addition of 50 µl of N-methyl-n-trimethylsilyltrifluoroacetamide with 1% 2,2,2-trifluoro-N-methyl-N-(trimethylsilyl)-acetamide, chlorotrimethylsilane (Thermo Scientific), vortexed for 10 secs followed by incubation at 80 °C for 15 mins and allowed to cool down at RT. After this, 50 µl of pyridine and 1 µl of the retention index solution were added and samples were vortexed for 10 secs. GC was performed in a TraceGOLD TG-5SILMS column with 30 m length, 0.25 mm inner diameter and 0.25 µm film thickness installed in a Trace Ultra gas chromatograph (both from

Thermo Scientific) with helium flow rate of 1.0 ml/min, then 1 µl of TMS-derivatised sample was injected into a split/splitless (SSL) injector at 250 °C using a surged splitless injection - splitless time of 30 secs and a surge pressure of 167 kPa. Temperature was increased from 70- up to 250 °C at a ramp rate of 50 °C/min followed by a ramp rate reduction of 10 °C/min reaching a final temperature of 330 °C which was maintained for 3.5 min. Eluting peaks were transferred at an auxiliary transfer temperature of 250 °C to a ITQ900-GC mass spectrometer (Thermo Scientific), with a filament delay of 5 min. Electron ionisation (70 V) was used with an emission current of 50 µA and an ion source that was held at 230 °C. The full scan mass range was 50-700 m/z with an automatic gain control (AGC) of 50%, and maximum ion time of 50 ms. Samples, blanks, pools and standards were loaded into the instrument. Analysis was performed with a TraceFinder v3.3 (Thermo Scientific) and sterols peaks were identified by matching the standards or by comparison to the NIST library.

Untargeted metabolomics by LC-MS

Mid log *L. mexicana* promastigotes (1×10^8) of all AmB-resistant lines alongside their parental wild-type were treated with AmB (Sigma®) at 5 x their EC_{50} for 15 mins as follows: the amount of AmB for lines selected in HOMEM was 0.3 µM for both parental lines (LmWT1 and LmWT2), 3 µM for AmBRcl.14 and AmBRcl.8, and 1.74 µM for AmBRB2. Both wild type (LmWT-DM) and AmBRA3-DM cultured in DM media were treated with AmB at 1.16 µM and 3.5 µM, respectively.

Metabolite extraction was performed by quenching parasite pellets by rapid cooling at 10°C in dry ice/ethanol bath, culture medium was removed by centrifugation at 1,250 g for 10 minutes at 4°C, then transferred centrifuged twice at 4,500 rpm for 10 minutes at 4°C with a wash in 1ml of cold PBS in between and the pellet was resuspended in 200 ul of monophasic chloroform/methanol/water (CMW 1:3:1) followed by 1 hour shaking (max speed) at 4°C

and a final spin at 13,000 rpm for 10 minutes at 4°C. Pooled sample was made by taking 10ul from each sample and blanks were also included. Samples were sealed after adding argon and stored at -80°C until analysis with LC-MS. Identification of metabolites for LC-MS was performed with a ZIC pHILIC column (150 mm × 4.6 mm, 5 µm column, Merck Sequant) coupled to high-resolution Thermo Orbitrap QExactive (Thermo Fisher Scientific) mass spectrometry in both positive and negative ionization modes. Samples were analysed in four replicates and data were processed in Glasgow Polyomics and provided as raw data. Identification of Liquid Chromatography-Mass Spectrometry (LC-MS) peaks involved mzmatch (161) run though PiMP (118) and IDEOM (162–164) and analysis was conducted in these latter software packages. LC-MS all raw files were submitted to the repository MetaboLights (165) (<https://www.ebi.ac.uk/metabolights/>) with project number MTBLS2744.

Genomic DNA extraction and sequencing.

Genomic DNA was extracted with the Nucleospin® Tissue kit (Macherey-Nagel) from polyene-resistant and wild-type mid-log promastigotes (1×10^7) washed twice in PBS at 1,250 g for 10 minutes. Library prep and sequencing were performed at Glasgow Polyomics using Illumina sequencer NextSeq 500 yielding 2 x 75 bp paired-end reads. Reference genomes of *L. mexicana* MHOM/GT/2001/U1103 and *L. infantum* JPCM5 (MCAN/ES/98/LLM-877) release 46 were obtained from the TriTrypDB (<https://tritrypdb.org>). Reads were mapped to their reference genome using BWA-MEM (166) and PCR duplicates were removed using GATK version 4.1.4.1 (167). Variant calling was performed using MuTect2 (168) with the default settings and variant annotation using snpEff (169). Filtered Variant Call Format (VCF) files corresponding to SNPs and indels with an impact on coding sequences were compared between wild type and resistant lines and

manually verified using the IGV 2.8.9 visualization tool (170) and eliminated if present in the parental line. Copy ratio alterations were detected using GATK (version 4.2.0.0). Reference genomes were divided into equally sized bins of 1,000 base pairs using PreprocessIntervals tool. Read counts in each bin were collected from alignment data using the CollectReadCounts tool. Copy ratios of a resistant sample over a matched non-resistant sample were obtained from read counts using CreateReadCountPanelOfNormals and DenoiseReadCounts tools. Copy number ratio was obtained by comparing the resistant lines with the corresponding copy number of wild type used as the base line. Genome contigs were segmented using ModelSegments tool from copy ratios of resistant and non-resistant samples. Amplified and deleted segments were identified using CallCopyRatioSegments tool with the default settings. Plots of denoised and segmented copy-ratios were generated using R software. Sequencing raw data was deposited at the NCBI National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov>) with project PRJNA763929 and PRJNA770503.

Other computational tools

BLAST search was performed against the NCBI database using default settings. ClustalW Omega was used to identify and align sequences. Protein queries-sequences were retrieved from TriTrypDB, Uniprot (<https://www.uniprot.org/>) and the Protein Data Bank (PDB) (<https://www.rcsb.org/>). Protein alignment viewers used were Clustal X2 (171). Protein-protein interaction map was created using STRING Database (<https://string-db.org>) (172–175).

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References

1. Burza S, Croft SL, Boelaert M. Leishmaniasis. The Lancet. 2018;392(10151):951–70.
2. Ghazanfar M, Malik MF. Sandfly and Leishmaniasis: A Review. Journal of Ecosystem & Ecography. 2016;6(3).
3. Karimkhani C, Wanga V, Naghavi P, Dellavalle RP, Naghavi M. Global burden of cutaneous leishmaniasis. The Lancet Infectious Diseases. 2017;17(3):264.
4. Das VNR, Siddiqui NA, Pal B, Lal CS, Verma N, Kumar A, et al. To evaluate efficacy and safety of amphotericin B in two different doses in the treatment of Post kala-azar dermal leishmaniasis (PKDL). PLoS ONE. 2017;12(3):1–13.

- 778 5. Hotez PJ, Bottazzi ME, Strych U. New Vaccines for the World's Poorest People. *Annual Review of*
779 *Medicine*. 2016 Jan 14;67(1):405–17.
- 780 6. Gangneux J-P, Sulahian A, Garin YJ-F, Farinotti R, Derouin F. Therapy of Visceral Leishmaniasis Due to
781 *Leishmania infantum*: Experimental Assessment of Efficacy of AmBisome. Vol. 40, *ANTIMICROBIAL*
782 *AGENTS AND CHEMOTHERAPY*. 1996.
- 783 7. Garcia A, Adler-Moore JP, Proffitt RT. Single-dose AmBisome (liposomal amphotericin B) as
784 prophylaxis for murine systemic candidiasis and histoplasmosis. *Antimicrobial Agents and*
785 *Chemotherapy*. 2000;44(9):2327–32.
- 786 8. Barrett MP, Croft SL. Management of trypanosomiasis and leishmaniasis. *British Medical Bulletin*.
787 2012;104(1):175–96.
- 788 9. Jain V, Jain K. Molecular targets and pathways for the treatment of visceral leishmaniasis. *Drug*
789 *Discovery Today*. 2018 Jan;23(1):161–70.
- 790 10. Ponte-Sucré A, Gamarro F, Dujardin JC, Barrett MP, López-Vélez R, García-Hernández R, et al. Drug
791 resistance and treatment failure in leishmaniasis: A 21st century challenge. Vol. 11, *PLoS Neglected*
792 *Tropical Diseases*. 2017. p. 1–24.
- 793 11. Mayers DL. Antimicrobial Drug Resistance: Mechanism of drug Resistance. Douglas Mayers, Jack S,
794 Ouellette M, Kaye K, Marchaim D, editors. Vol. 1. Springer US; 2017. 299.
- 795 12. Yardley V, Croft SL. Activity of liposomal amphotericin B against experimental cutaneous leishmaniasis.
796 *Antimicrobial Agents and Chemotherapy*. 1997;41(4):752–6.
- 797 13. Fairlamb AH, Gow NAR, Matthews KR, Waters AP. Drug resistance in eukaryotic microorganisms. Vol.
798 1, *Nature Microbiology*. 2016.
- 799 14. Lopes S, Castanho MARB. Revealing the orientation of nystatin and amphotericin B in lipidic multilayers
800 by UV-Vis linear dichroism. *Journal of Physical Chemistry B*. 2002;106(29):7278–82.
- 801 15. Kristanc L, Božič B, Jokhadar ŠZ, Dolenc MS, Gomišček G. The pore-forming action of polyenes: From
802 model membranes to living organisms. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 2019
803 Feb;1861(2):418–30.
- 804 16. Bolard J. How do the polyene macrolide antibiotics affect the cellular membrane properties?
805 1986;864:257–304.
- 806 17. Chattopadhyay A, Jafurulla M. A novel mechanism for an old drug: Amphotericin B in the treatment of
807 visceral leishmaniasis. *Biochemical and Biophysical Research Communications*. 2011;416(1–2):7–12.
- 808 18. Ostrosky-Zeichner L, Casadevall A, Galgiani JN, Odds FC, Rex JH. An insight into the antifungal
809 pipeline: Selected new molecules and beyond. *Nature Reviews Drug Discovery*. 2010;9(9):719–27.
- 810 19. Vandeputte P, Ferrari S, Coste AT. Antifungal resistance and new strategies to control fungal infections.
811 *International Journal of Microbiology*. 2012;2012.
- 812 20. Cowen LE. The evolution of fungal drug resistance: Modulating the trajectory from genotype to
813 phenotype. *Nature Reviews Microbiology*. 2008;6(3):187–98.
- 814 21. Cohen BE. The Role of Signaling via Aqueous Pore Formation in Resistance Responses to Amphotericin
815 B. *Antimicrobial Agents and Chemotherapy*. 2016 Sep;60(9):5122–9.
- 816 22. Takemoto K, Yamamoto Y, Ueda Y, Sumita Y, Yoshida K, Niki Y. Comparative study on the efficacy of
817 amBisome and fungizone in a mouse model of pulmonary aspergillosis. *Journal of Antimicrobial*
818 *Chemotherapy*. 2006;57(4):724–31.
- 819 23. Glasser JS, Murray CK. Case report: Central nervous system toxicity associated with liposomal
820 amphotericin B therapy for cutaneous leishmaniasis. *American Journal of Tropical Medicine and*
821 *Hygiene*. 2011 Apr;84(4):566–8.
- 822 24. Nath P, Basher A, Harada M, Sarkar S, Selim S, Maude RJ, et al. Immediate hypersensitivity reaction
823 following liposomal amphotericin-B (AmBisome) infusion. *Tropical Doctor*. 2014;44(4):241–2.

- 824 25. Berman JD, Badaro R, Thakur CP, Wasunna KM, Behbehani K, Davidson R, et al. Efficacy and safety
825 of liposomal amphotericin B (AmBisome) for visceral leishmaniasis in endemic developing countries*.
826 1998.
- 827 26. Sundar S, Sinha K, Rai M, Verma K, Nawin K, Alam S, et al. Comparison of short-course multidrug
828 treatment with standard therapy for visceral leishmaniasis in India: an open-label, non-inferiority,
829 randomised controlled trial. *www.thelancet.com*. 2011;377.
- 830 27. den Boer M, Argaw D, Jannin J, Alvar J. Leishmaniasis impact and treatment access. *Clinical*
831 *Microbiology and Infection*. 2011;17(10):1471–7.
- 832 28. Bhattacharya P, Ali N. Treatment of visceral leishmaniasis: anomalous pricing and distribution of
833 AmBisome and emergence of an indigenous liposomal amphotericin B, FUNGISOME. *Journal of*
834 *Parasitic Diseases*. 2016;40.
- 835 29. Balasegaram M, Ritmeijer K, Lima MA, Burza S, Ortiz Genovese G, Milani B, et al. Liposomal
836 amphotericin B as a treatment for human leishmaniasis. *Expert Opinion on Emerging Drugs*. 2012 Dec
837 20;17(4):493–510.
- 838 30. Alves F, Bilbe G, Blesson S, Goyal V, Monnerat S, Mowbray C, et al. Recent Development of Visceral
839 Leishmaniasis Treatments: Successes, Pitfalls, and Perspectives. *Clinical Microbiology Reviews*. 2018
840 Aug 29;31(4):1–30.
- 841 31. WHO. Department of Control of Neglected Tropical Diseases Sustaining the drive to overcome the global
842 impact of neglected tropical diseases Second WHO report on neglected tropical diseases. 2013.
- 843 32. MSF. MSF statement in response to Gilead donation of AmBisome for visceral leishmaniasis | Médecins
844 Sans Frontières Access Campaign [Internet]. 2011 [cited 2021 Apr 10]. Available from:
845 <https://msfaccess.org/msf-statement-response-gilead-donation-ambisome-visceral-leishmaniasis>
- 846 33. Davidson RN, Scott A, Maini M, Bryceson ADM, Croft SL. Liposomal amphotericin B in drug-resistant
847 visceral leishmaniasis. *The Lancet*. 1991;
- 848 34. del Rosal T, Artigao FB, Miguel MJG, de Lucas R, del Castillo F. Successful treatment of childhood
849 cutaneous leishmaniasis with liposomal amphotericin B: Report of two cases. *Journal of Tropical*
850 *Pediatrics*. 2009 Aug 5;56(2):122–4.
- 851 35. Lambertucci JR, Cristina L, Silva S. Mucocutaneous leishmaniasis treated with liposomal amphotericin B
852 Leishmaniose cutâneo mucosa tratada com anfotericina B lipossomal IMAGENS EM DIP/IMAGES IN
853 INFECTIOUS DISEASE A. Vol. 41, Revista da Sociedade Brasileira de Medicina Tropical. 2008.
- 854 36. Yardley V, Croft SL. A comparison of the activities of three amphotericin B lipid formulations against
855 experimental visceral and cutaneous leishmaniasis. *International Journal of Antimicrobial Agents*.
856 2000;13(4):243–8.
- 857 37. Gill N, Pandey D, Roy N. Kala-azar in India – progress and challenges towards its elimination as a public
858 health problem. 2021;267–79.
- 859 38. Rijal S, Sundar S, Mondal D, Das P, Alvar J, Boelaert M. Eliminating visceral leishmaniasis in South
860 Asia: The road ahead. Vol. 364, *BMJ (Online)*. 2019.
- 861 39. Ruiz-Postigo JA, Jain S, Ruiz-postigo JA, Jain S, Asia E. Global leishmaniasis a baseline for the 2030
862 roadmap Surveillance mondiale de la leishmaniose : 2019-2020 , une période de référence pour la feuille
863 de route à l ' horizon 2030. 2021;2019–20.
- 864 40. WHO. Ending the neglect to attain the Sustainable Development Goals – A road map for neglected
865 tropical diseases 2021–2030. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0
866 IGO. [Internet]. 2021 [cited 2021 Apr 10]. Available from:
867 https://www.who.int/neglected_diseases/Ending-the-neglect-to-attain-the-SDGs--NTD-Roadmap.pdf
- 868 41. Malecela MN, Ducker C. A road map for neglected tropical diseases 2021-2030. *Trans R Soc Trop Med*
869 *Hyg*. 2021;115:121–3.
- 870 42. Ellis D. Amphotericin B: Spectrum and resistance. *Journal of Antimicrobial Chemotherapy*.
871 2002;49(SUPL. S1):7–10.

- 872 43. Mesa-Arango AC, Scorzoni L, Zaragoza O. It only takes one to do many jobs: Amphotericin B as
873 antifungal and immunomodulatory drug. *Frontiers in Microbiology*. 2012;3(AUG):1–10.
- 874 44. Sangalli-Leite F, Scorzoni L, Mesa-Arango AC, Casas C, Herrero E, Soares Mendes Gianinni MJ, et al.
875 Amphotericin B mediates killing in *Cryptococcus neoformans* through the induction of a strong oxidative
876 burst. *Microbes and Infection*. 2011;13(5):457–67.
- 877 45. Posch W, Blatzer M, Wilflingseder D, Lass-Flörl C. *Aspergillus terreus*: Novel lessons learned on
878 amphotericin B resistance. Vol. 56, *Medical Mycology*. Oxford University Press; 2018. p. S73–82.
- 879 46. Moosa M-YS. Resistance to amphotericin B does not emerge during treatment for invasive aspergillosis.
880 *Journal of Antimicrobial Chemotherapy*. 2002 Jan 1;49(1):209–13.
- 881 47. Lachaud L, Bourgeois N, Plourde M, Leprohon P, Bastien P, Ouellette M. Parasite Susceptibility to
882 Amphotericin B in Failures of Treatment for Visceral Leishmaniasis in Patients Coinfected with HIV
883 Type 1 and *Leishmania infantum*. *Clinical Infectious Diseases*. 2009;48(2):e16–22.
- 884 48. Burza S, Sinha PK, Mahajan R, Sanz MG, Lima MA, Mitra G, et al. Post Kala-Azar dermal leishmaniasis
885 following treatment with 20 mg/kg liposomal amphotericin B (Ambisome) for primary visceral
886 leishmaniasis in Bihar, India. *PLoS neglected tropical diseases*. 2014;8(1):e2611.
- 887 49. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. *Reviews of*
888 *Infectious Diseases*. 1990;12(2):308–29.
- 889 50. Bolard J. How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochimica*
890 *et biophysica acta*. 1986 Dec 22;864(3–4):257–304.
- 891 51. Marty A, Finkelstein A. Pores formed in lipid bilayer membranes by nystatin, Differences in its one-sided
892 and two-sided action. *Journal of General Physiology*. 1975 Apr 1;65(4):515–26.
- 893 52. Serhan G, Stack CM, Perrone GG, Morton CO. The polyene antifungals, amphotericin B and nystatin,
894 cause cell death in *Saccharomyces cerevisiae* by a distinct mechanism to amphibian-derived antimicrobial
895 peptides. *Annals of Clinical Microbiology and Antimicrobials*. 2014;13(1):1–4.
- 896 53. Te Welscher YM, Ten Napel HH, Balagué MM, Souza CM, Riezman H, De Kruijff B, et al. Natamycin
897 blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *Journal*
898 *of Biological Chemistry*. 2008;283(10):6393–401.
- 899 54. Te Welscher YM, Jones L, Van Leeuwen MR, Dijksterhuis J, De Kruijff B, Eitzen G, et al. Natamycin
900 inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. *Antimicrobial*
901 *Agents and Chemotherapy*. 2010;54(6):2618–25.
- 902 55. Tewary P, Veena K, Pucadyil TJ, Chattopadhyay A, Madhubala R. The sterol-binding antibiotic nystatin
903 inhibits entry of non-opsonized *Leishmania donovani* into macrophages. *Biochemical and Biophysical*
904 *Research Communications*. 2006;339(2):661–6.
- 905 56. Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS, Tuttle MD, et al. Amphotericin forms an
906 extramembranous and fungicidal sterol sponge. *Nature Chemical Biology*. 2014;10(5):400–6.
- 907 57. Hsueh C-C, Feingold DS. Selective Membrane Toxicity of the Polyene Antibiotics: Studies on Lecithin
908 Membrane Models (Liposomes). *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*. 1973.
- 909 58. Smith SJ, Crowley JH, Parks LW. Transcriptional regulation by ergosterol in the yeast *Saccharomyces*
910 *cerevisiae*. *Molecular and Cellular Biology*. 1996;16(10):5427–32.
- 911 59. Veen M, Lang C. Interactions of the ergosterol biosynthetic pathway with other lipid pathways. Vol. 33,
912 *Biochemical Society Transactions*. 2005.
- 913 60. Purkait B, Kumar A, Nandi N, Sardar AH, Das S, Kumar S, et al. Mechanism of Amphotericin B
914 Resistance in Clinical Isolates of *Leishmania donovani*. *Antimicrobial Agents and Chemotherapy*. 2012
915 Feb;56(2):1031–41.
- 916 61. Giorgio C. Flow cytometric assessment of amphotericin B susceptibility in. 1999;71–6.

- 917 62. Durand R, Paul M, Pratlong F, Rivollet D, Dubreuil-Lemaire ML, Houin R, et al. Leishmania infantum:
918 Lack of parasite resistance to amphotericin B in a clinically resistant visceral leishmaniasis. Antimicrobial
919 Agents and Chemotherapy. 1998;42(8):2141–3.
- 920 63. Srivastava P, Prajapati VK, Rai M, Sundar S. Unusual case of resistance to amphotericin B in visceral
921 leishmaniasis in a region in India where leishmaniasis is not endemic. Journal of Clinical Microbiology.
922 2011;49(8):3088–91.
- 923 64. Chakravarty J, Sundar S. Drug resistance in leishmaniasis. Journal of Global Infectious Diseases.
924 2010;2(2):167.
- 925 65. de Moura TR, Santos MLB, Braz JM, Santos LFVC, Aragão MT, de Oliveira FA, et al. Cross-resistance
926 of Leishmania infantum isolates to nitric oxide from patients refractory to antimony treatment, and greater
927 tolerance to antileishmanial responses by macrophages. Parasitology Research. 2016;115(2):713–21.
- 928 66. Eichenberger A, Buechi AE, Neumayr A, Hatz C, Rauch A, Huguenot M, et al. A severe case of visceral
929 leishmaniasis and liposomal amphotericin B treatment failure in an immunosuppressed patient 15 years
930 after exposure. BMC Infectious Diseases. 2017 Jan 17;17(1).
- 931 67. Tamiru A, Tigabu B, Yifru S, Diro E, Hailu A. Safety and efficacy of liposomal amphotericin B for
932 treatment of complicated visceral leishmaniasis in patients without HIV, North-West Ethiopia. BMC
933 Infectious Diseases. 2016 Oct 10;16(1):548.
- 934 68. Ritmeijer K, Ter Horst R, Chane S, Aderie EM, Piening T, Collin SM, et al. Limited effectiveness of
935 high-dose liposomal amphotericin B (AmBisome) for treatment of visceral leishmaniasis in an ethiopian
936 population with high HIV prevalence. Clinical Infectious Diseases. 2011 Dec 15;53(12).
- 937 69. Sardi de CO, Silva DR, Soares Mendes-Giannini MJ, Rosalen PL. Candida auris: Epidemiology, risk
938 factors, virulence, resistance, and therapeutic options. Microbial Pathogenesis. 2018 Dec 1;125:116–21.
- 939 70. Branco J, Ola M, Silva RM, Fonseca E, Gomes NC, Martins-Cruz C, et al. Impact of ERG3 mutations and
940 expression of ergosterol genes controlled by UPC2 and NDT80 in Candida parapsilosis azole resistance.
941 Clinical Microbiology and Infection. 2017 Aug;23(8):575.e1-575.e8.
- 942 71. Joseph-Horne T, Manning NJ, Hollomon D, Kelly SL. Defective sterol $\Delta 5(6)$ desaturase as a cause of
943 azole resistance in Ustilago maydis. FEMS Microbiology Letters. 1995 Mar;127(1–2):29–34.
- 944 72. Kelly SL, Lamb DC, Taylor M, Corran AJ, Baldwin BC, Powderly WG. Resistance to amphotericin B
945 associated with defective sterol $\Delta 8,7$, isomerase in a Cryptococcus neoformans strain from an AIDS
946 patient. FEMS Microbiology Letters. 1994 Sep 1;122(1–2):39–42.
- 947 73. Martel CM, Parker JE, Bader O, Weig M, Gross U, Warrilow AGS, et al. A clinical isolate of Candida
948 albicans with mutations in ERG11 (encoding sterol 14 α -demethylase) and ERG5 (encoding C22
949 desaturase) is cross resistant to azoles and amphotericin B. Antimicrobial agents and chemotherapy. 2010
950 Sep;54(9):3578–83.
- 951 74. Paterson PJ, Seaton S, Prentice HG, Kibbler CC. Treatment failure in invasive aspergillosis: Susceptibility
952 of deep tissue isolates following treatment with amphotericin B. Journal of Antimicrobial Chemotherapy.
953 2003 Nov;52(5):873–6.
- 954 75. Young LY, Hull CM, Heitman J. Disruption of ergosterol biosynthesis confers resistance to amphotericin
955 B in Candida lusitanae. Antimicrobial Agents and Chemotherapy. 2003;47(9):2717–24.
- 956 76. Sokol-Anderson ML, Brajtburg J, Medoff G. Amphotericin B-induced oxidative damage and killing of
957 Candida albicans. The Journal of infectious diseases. 1986 Jul;154(1):76–83.
- 958 77. Vincent BM, Lancaster AK, Scherz-Shouval R, Whitesell L, Lindquist S. Fitness Trade-offs Restrict the
959 Evolution of Resistance to Amphotericin B. Mitchell AP, editor. PLoS Biology. 2013 Oct
960 29;11(10):e1001692.
- 961 78. Vahedi Shahandashti R, Lass-Flörl C. Antifungal resistance in Aspergillus terreus: A current scenario.
962 Fungal Genetics and Biology. 2019;131(June):103247.

- 963 79. dos Santos RAC, Steenwyk JL, Rivero-Menendez O, Mead ME, Silva LP, Bastos RW, et al. Genomic and
964 Phenotypic Heterogeneity of Clinical Isolates of the Human Pathogens *Aspergillus fumigatus*, *Aspergillus*
965 *lentulus*, and *Aspergillus fumigatiaffinis*. *Frontiers in Genetics*. 2020;11(May):1–17.
- 966 80. Satragno D, Faral-Tello P, Canneva B, Verger L, Lozano A, Vitale E, et al. Autochthonous outbreak and
967 expansion of canine visceral Leishmaniasis, Uruguay. *Emerging Infectious Diseases*. 2017;23(3):536–8.
- 968 81. Faral-Tello P, Greif G, Satragno D, Basmadjian Y, Robello C. *Leishmania infantum* isolates exhibit high
969 infectivity and reduced susceptibility to amphotericin B. *RSC Medicinal Chemistry*. 2020;11(8):913–8.
- 970 82. Franco-Muñoz C, Manjarré S-Estremor M, Ovalle-Bracho C. Intraspecies differences in natural
971 susceptibility to amphotericin B of clinical isolates of *Leishmania* subgenus *Viannia*. 2018;
- 972 83. Purkait B, Singh R, Wasnik K, Das S, Kumar A, Paine M, et al. Up-regulation of silent information
973 regulator 2 (Sir2) is associated with amphotericin B resistance in clinical isolates of *Leishmania donovani*.
974 *Journal of Antimicrobial Chemotherapy*. 2015 May 1;70(5):1343–56.
- 975 84. Alcazar-Fuoli L, Mellado E. Ergosterol biosynthesis in *Aspergillus fumigatus*: Its relevance as an
976 antifungal target and role in antifungal drug resistance. *Frontiers in Microbiology*. 2012;3(JAN):1–6.
- 977 85. Kontoyiannis DP, Lewis RE. Antifungal drug resistance of pathogenic fungi. *The Lancet*. 2002 Mar
978 30;359(9312):1135–44.
- 979 86. Kaur G, Rajput B. Comparative analysis of the omics technologies used to study antimonial, amphotericin
980 b, and pentamidine resistance in leishmania. *Journal of Parasitology Research*. 2014;2014.
- 981 87. Brotherton MC, Bourassa S, Légaré D, Poirier GG, Droit A, Ouellette M. Quantitative proteomic analysis
982 of amphotericin B resistance in *Leishmania infantum*. *International Journal for Parasitology: Drugs and*
983 *Drug Resistance*. 2014;4(2):126–32.
- 984 88. Singh J, Khan MI, Singh Yadav SP, Srivastava A, Sinha KK, Ashish, et al. L-Asparaginase of *Leishmania*
985 *donovani*: Metabolic target and its role in Amphotericin B resistance. *International Journal for*
986 *Parasitology: Drugs and Drug Resistance*. 2017;7(3):337–49.
- 987 89. Singh K, Ali V, Pratap Singh K, Gupta P, Suman SS, Ghosh AK, et al. Deciphering the interplay between
988 cysteine synthase and thiol cascade proteins in modulating Amphotericin B resistance and survival of
989 *Leishmania donovani* under oxidative stress. *Redox Biology*. 2017;
- 990 90. Kumar A, Das S, Purkait B, Sardar AH, Ghosh AK, Dikhit MR, et al. Ascorbate peroxidase, a key
991 molecule regulating amphotericin B resistance in clinical isolates of *Leishmania donovani*. *Antimicrobial*
992 *Agents and Chemotherapy*. 2014;58(10):6172–84.
- 993 91. Suman SS, Equbal A, Zaidi A, Ansari MdY, Singh KP, Singh K, et al. Up-regulation of cytosolic
994 trypanothione in Amp B resistant isolates of *Leishmania donovani* and its interaction with cytosolic
995 trypanothione peroxidase. *Biochimie*. 2016 Feb;121:312–25.
- 996 92. Purkait B, Singh R, Wasnik K, Das S, Kumar A, Paine M, et al. Up-regulation of silent information
997 regulator 2 (Sir2) is associated with amphotericin B resistance in clinical isolates of *Leishmania donovani*.
998 *Journal of Antimicrobial Chemotherapy*. 2015 May;70(5):1343–56.
- 999 93. Fernandez-Prada C, Vincent IM, Brotherton MC, Roberts M, Roy G, Rivas L, et al. Different Mutations
1000 in a P-type ATPase Transporter in *Leishmania* Parasites are Associated with Cross-resistance to Two
1001 Leading Drugs by Distinct Mechanisms. *PLoS Neglected Tropical Diseases*. 2016;10(12):1–20.
- 1002 94. Seifert K, Matu S, Pérez-Victoria FJ, Castanys S, Gamarro F, Croft SL. Characterisation of *Leishmania*
1003 *donovani* promastigotes resistant to hexadecylphosphocholine (miltefosine). *International Journal of*
1004 *Antimicrobial Agents*. 2003;22(4):380–7.
- 1005 95. Mwenechanya R, Kovářová J, Dickens NJ, Mudaliar M, Herzyk P, Vincent IM, et al. Sterol 14 α -
1006 demethylase mutation leads to amphotericin B resistance in *Leishmania mexicana*. Raper J, editor. *PLOS*
1007 *Neglected Tropical Diseases*. 2017 Jun 16;11(6):e0005649.
- 1008 96. Pountain AW, Weidt SK, Regnault C, Bates PA, Donachie AM, Dickens NJ, et al. Genomic instability at
1009 the locus of sterol C24-methyltransferase promotes amphotericin B resistance in *Leishmania* parasites.
1010 2019;

- 1011 97. Mbongo N, Loiseau PM, Billion MA, Malka Robert-Gero. Mechanism of Amphotericin B Resistance in
1012 *Leishmania donovani* Promastigotes These include : Mechanism of Amphotericin B Resistance in
1013 *Leishmania donovani* Promastigotes. *Antimicrob Agents Chemother.* 1998;42(2):352–357.
- 1014 98. Pourshafie M, Morand S, Virion A, Rakotomanga M, Dupuy C, Loiseau PM. Cloning of S-adenosyl-L-
1015 methionine:C-24- Δ -sterol-methyltransferase (ERG6) from *Leishmania donovani* and characterization of
1016 mRNAs in wild-type and amphotericin B-resistant promastigotes. *Antimicrobial Agents and*
1017 *Chemotherapy.* 2004;48(7):2409–14.
- 1018 99. Rastrojo A, García-Hernández R, Vargas P, Camacho E, Corvo L, Imamura H, et al. Genomic and
1019 transcriptomic alterations in *Leishmania donovani* lines experimentally resistant to antileishmanial drugs.
1020 *International Journal for Parasitology: Drugs and Drug Resistance.* 2018;8(2):246–64.
- 1021 100. Mondelaers A, Sanchez-Cañete MP, Hendrickx S, Eberhardt E, Garcia-Hernandez R, Lachaud L, et al.
1022 Genomic and Molecular Characterization of Miltefosine Resistance in *Leishmania infantum* Strains with
1023 Either Natural or Acquired Resistance through Experimental Selection of Intracellular Amastigotes.
1024 Satoskar AR, editor. *PLOS ONE.* 2016 Apr 28;11(4):e0154101.
- 1025 101. Pountain AW, Weidt SK, Regnault C, Bates PA, Donachie AM, Dickens NJ, et al. Genomic instability at
1026 the locus of sterol C24-methyltransferase promotes amphotericin B resistance in *Leishmania* parasites.
1027 Pollastri MP, editor. *PLOS Neglected Tropical Diseases.* 2019 Feb 4;13(2):e0007052.
- 1028 102. Shaw CD, Lonchamp J, Downing T, Imamura H, Freeman TM, Cotton JA, et al. In vitro selection of
1029 miltefosine resistance in promastigotes of *Leishmania donovani* from Nepal: Genomic and metabolomic
1030 characterization. *Molecular Microbiology.* 2016;99(6):1134–48.
- 1031 103. Xu W, Hsu F-F, Baykal E, Huang J, Zhang K. Sterol Biosynthesis Is Required for Heat Resistance but
1032 Not Extracellular Survival in *Leishmania*. *PLoS Pathog.* 2014;10(10):1004427.
- 1033 104. McCall LI, El Aroussi A, Choi JY, Vieira DF, De Muylder G, Johnston JB, et al. Targeting Ergosterol
1034 Biosynthesis in *Leishmania donovani*: Essentiality of Sterol 14 α -demethylase. *PLoS Neglected*
1035 *Tropical Diseases.* 2015;9(3):1–17.
- 1036 105. Mukherjee S, Moitra S, Xu W, Hernandez V, Zhang K. Sterol 14- α -demethylase is vital for mitochondrial
1037 functions and stress tolerance in *Leishmania major*. *PLoS Pathogens.* 2020 Aug 1;16(8).
- 1038 106. Mukherjee S, Xu W, Hsu F-F, Patel J, Huang J, Zhang K. Sterol methyltransferase is required for optimal
1039 mitochondrial function and virulence in *Leishmania major*. *Molecular Microbiology.* 2019 Jan;111(1):65–
1040 81.
- 1041 107. Ning Y, Frankfater C, Hsu F-F, Soares RP, Cardoso CA, Nogueira PM, et al. Lathosterol Oxidase (Sterol
1042 C-5 Desaturase) Deletion Confers Resistance to Amphotericin B and Sensitivity to Acidic Stress in
1043 *Leishmania major* . *mSphere.* 2020 Jul 1;5(4).
- 1044 108. Bekersky I, Fielding RM, Dressler DE, Lee JW, Buell DN, Walsh TJ. Plasma protein binding of
1045 amphotericin B and pharmacokinetics of bound versus unbound amphotericin B after administration of
1046 intravenous liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate. *Antimicrobial*
1047 *Agents and Chemotherapy.* 2002;46(3):834–40.
- 1048 109. Brajtburg J, Elberg S, Bolard J, Kobayashi GS, Levy RA, Ostlund RE, et al. Interaction of Plasma
1049 Proteins and Lipoproteins with Amphotericin B. Vol. 149, *THE JOURNAL OF INFECTIOUS*
1050 *DISEASES* •. 1984.
- 1051 110. Choi JY, Podust LM, Roush WR. Drug Strategies Targeting CYP51 in Neglected Tropical Diseases.
1052 2014;
- 1053 111. Emami S, Tavangar P, Keighobadi M. An overview of azoles targeting sterol 14 α -demethylase for
1054 antileishmanial therapy. Vol. 135, *European Journal of Medicinal Chemistry.* 2017. p. 241–59.
- 1055 112. De Macedo-Silva ST, Urbina JA, De Souza W, Rodrigues JCF. In vitro activity of the antifungal azoles
1056 itraconazole and posaconazole against *Leishmania amazonensis*. *PLoS ONE.* 2013;8(12).
- 1057 113. Zeiman E, Greenblatt CL, Elgavish S, Khozin-Goldberg I, Golenser J. Mode of Action of Fenarimol
1058 Against *Leishmania* Spp. *Journal of Parasitology.* 2008;94(1):280–6.

- 1059 114. Mina JGM, Charlton RL, Alpizar-Sosa E, Escrivani DO, Brown C, Alqaisi A, et al. Antileishmanial
1060 Chemotherapy through Clemastine Fumarate Mediated Inhibition of the Leishmania Inositol
1061 Phosphorylceramide Synthase. *ACS Infectious Diseases*. 2021;7(1):47–63.
- 1062 115. Farjami E, Clima L, Gothelf K V., Ferapontova EE. DNA interactions with a Methylene Blue redox
1063 indicator depend on the DNA length and are sequence specific. *The Analyst*. 2010 May 24;135(6):1443.
- 1064 116. Kelner MJ, Alexander NM. THE JOURNAL OF BIOLOGICAL CHEMISTRY Methylene Blue Directly
1065 Oxidizes Glutathione without the Intermediate Formation of Hydrogen Peroxide. Vol. 260. 1985.
- 1066 117. Buchholz K, Schirmer RH, Eubel JK, Akoachere MB, Dandekar T, Becker K, et al. Interactions of
1067 methylene blue with human disulfide reductases and their orthologues from *Plasmodium falciparum*.
1068 Antimicrobial agents and chemotherapy. 2008 Jan;52(1):183–91.
- 1069 118. Gloaguen Y, Morton F, Daly R, Gurden R, Rogers S, Wandy J, et al. PiMP my metabolome: an
1070 integrated, web-based tool for LC-MS metabolomics data. Wren J, editor. *Bioinformatics*. 2017 Dec
1071 15;33(24):4007–9.
- 1072 119. Ghosh AK, Sardar AH, Mandal A, Saini S, Abhishek K, Kumar A, et al. Metabolic reconfiguration of the
1073 central glucose metabolism: a crucial strategy of *Leishmania donovani* for its survival during oxidative
1074 stress. *The FASEB Journal*. 2015 May 17;29(5):2081–98.
- 1075 120. Kovářová J, Pountain AW, Wildridge D, Weidt S, Bringaud F, Burchmore RJS, et al. Deletion of
1076 transketolase triggers a stringent metabolic response in promastigotes and loss of virulence in amastigotes
1077 of *Leishmania mexicana*. Vol. 14, *PLoS Pathogens*. 2018. 1–31.
- 1078 121. Maugeri DA, Cazzulo JJ. The pentose phosphate pathway in *Trypanosoma cruzi*. *FEMS Microbiology*
1079 *Letters*. 2004 May 1;234(1):117–23.
- 1080 122. Gray KC, Palacios DS, Dailey I, Endo MM, Uno BE, Wilcock BC, et al. Amphotericin primarily kills
1081 yeast by simply binding ergosterol. *Proceedings of the National Academy of Sciences*. 2012;109(7):2234–
1082 9.
- 1083 123. Al-Mohammed HI, Chance ML, Bates PA. Production and characterization of stable amphotericin-
1084 resistant amastigotes and promastigotes of *Leishmania mexicana*. *Antimicrobial Agents and*
1085 *Chemotherapy*. 2005;49(8):3274–80.
- 1086 124. Andrade-Neto VV, Pereira TM, Do Canto-Cavalheiro M, Caio Torres-Santos E. Imipramine alters the
1087 sterol profile in *Leishmania amazonensis* and increases its sensitivity to miconazole. *Parasites and*
1088 *Vectors*. 2016;9(1):1–8.
- 1089 125. Andrade-Neto VV, Cunha-Júnior EF, Do Canto-Cavalheiro MM, Atella GC, De Almeida Fernandes T,
1090 Costa PRR, et al. Antileishmanial activity of ezetimibe: Inhibition of sterol biosynthesis, in vitro synergy
1091 with azoles, and efficacy in experimental cutaneous leishmaniasis. *Antimicrobial Agents and*
1092 *Chemotherapy*. 2016;60(11):6844–52.
- 1093 126. Brooks DN, Weber R V, Chao JD, Rinker BD, Zoldos J, Robichaux MR, et al. Processed nerve allografts
1094 for peripheral nerve reconstruction: a multicenter study of utilization and outcomes in sensory, mixed, and
1095 motor nerve reconstructions. *Microsurgery*. 2012 Jan;32(1):1–14.
- 1096 127. Bansal R, Sen SS, Muthuswami R, Madhubala R. A Plant like Cytochrome P450 Subfamily CYP710C1
1097 Gene in *Leishmania donovani* Encodes Sterol C-22 Desaturase and its Over-expression Leads to
1098 Resistance to Amphotericin B. Nakhasi HL, editor. *PLOS Neglected Tropical Diseases*. 2019 Apr
1099 3;13(4):e0007260.
- 1100 128. Roberts CW, McLeod R, Rice DW, Ginger M, Chance ML, Goad LJ. Fatty acid and sterol metabolism:
1101 Potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. *Molecular and*
1102 *Biochemical Parasitology*. 2003;126(2):129–42.
- 1103 129. Yao C, Dixit UG, Barker JH, Teesch LM, Love-Homan L, Donelson JE, et al. Attenuation of *Leishmania*
1104 *infantum* chagasi metacyclic promastigotes by sterol depletion. Adams JH, editor. *Infection and*
1105 *Immunity*. 2013 Jul;81(7):2507–17.
- 1106 130. Yao C, Wilson ME. Dynamics of sterol synthesis during development of *Leishmania* spp. parasites to
1107 their virulent form. *Parasites and Vectors*. 2016 Dec 12;9(1):1–12.

- 1108 131. Andrade-Neto VV, Nicole Theodore Cicco N, Ferreira Cunha-Junior E, Marcuzzo Canto-Cavalheiro M,
1109 Correa Atella G, Caio Torres-Santos E. The pharmacological inhibition of sterol biosynthesis in
1110 Leishmania is counteracted by enhancement of LDL endocytosis. *Acta Tropica*. 2011;119:194–8.
- 1111 132. Ginger ML, Chance ML, Sadler IH, Goad LJ. The biosynthetic incorporation of the intact leucine skeleton
1112 into sterol by the trypanosomatid *Leishmania mexicana*. *The Journal of biological chemistry*. 2001 Apr
1113 13;276(15):11674–82.
- 1114 133. Ghosh M, Roy K, Das Mukherjee D, Chakrabarti G, Roy Choudhury K, Roy S. *Leishmania donovani*
1115 Infection Enhances Lateral Mobility of Macrophage Membrane Protein Which Is Reversed by Liposomal
1116 Cholesterol. Jardim A, editor. *PLoS Neglected Tropical Diseases*. 2014 Dec 4;8(12):e3367.
- 1117 134. Equbal A, Suman SS, Anwar S, Singh KP, Zaidi A, Hasan Sardar A, et al. Stage-Dependent Expression
1118 and Up-Regulation of Trypanothione Synthetase in Amphotericin B Resistant *Leishmania donovani*.
1119 2014;
- 1120 135. Yao C, Chen Y, Sudan B, Donelson JE, Wilson ME. *Leishmania chagasi*: Homogenous metacyclic
1121 promastigotes isolated by buoyant density are highly virulent in a mouse model. *Experimental*
1122 *Parasitology*. 2008 Jan;118(1):129–33.
- 1123 136. Krieger S, Schwarz W, Ariyanayagam MR, Fairlamb AH, Krauth-Siegel RL, Clayton C. Trypanosomes
1124 lacking trypanothione reductase are avirulent and show increased sensitivity to oxidative stress. *Molecular*
1125 *Microbiology*. 2000 Apr 5;35(3):542–52.
- 1126 137. Vargas J, López M, Escovar J, Fernández J. Evaluacion por Western Blot, Inmunofluorescencia Indirecta
1127 y ELISA de Perros Infectados con *Leishmania (Leishmania) infantum*. *Revista de Salud Pública*.
1128 2009;11(4):641–52.
- 1129 138. Diro E, Blesson S, Edwards T, Ritmeijer K, Fikre H, Admassu H, et al. A randomized trial of AmBisome
1130 monotherapy and AmBisome and miltefosine combination to treat visceral leishmaniasis in HIV co-
1131 infected patients in Ethiopia. Carvalho EM, editor. *PLOS Neglected Tropical Diseases*. 2019 Jan
1132 17;13(1):e0006988.
- 1133 139. Ubeda J-M, Légaré D, Raymond F, Ouameur AA, Boisvert S, Rigault P, et al. Modulation of gene
1134 expression in drug resistant *Leishmania* is associated with gene amplification, gene deletion and
1135 chromosome aneuploidy. *Genome biology*. 2008;9(7):R115.
- 1136 140. Pountain AW, Weidt SK, Regnault C, Bates PA, Donachie AM, Dickens NJ, et al. Genomic instability at
1137 the locus of sterol C24-methyltransferase promotes amphotericin B resistance in *Leishmania* parasites.
1138 2019;
- 1139 141. Lamb DC, Kelly DE, White TC, Kelly SL. The R467K amino acid substitution in *Candida albicans* sterol
1140 14 α - demethylase causes drug resistance through reduced affinity. *Antimicrobial Agents and*
1141 *Chemotherapy*. 2000;44(1):63–7.
- 1142 142. Dunkel N, Liu TT, Barker KS, Homayouni R, Morschhäuser J, Rogers PD. A gain-of-function mutation
1143 in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased
1144 fluconazole resistance in a clinical *Candida albicans* isolate. *Eukaryotic Cell*. 2008;7(7):1180–90.
- 1145 143. Singh K, Ali V, Pratap Singh K, Gupta P, Suman SS, Ghosh AK, et al. Deciphering the interplay between
1146 cysteine synthase and thiol cascade proteins in modulating Amphotericin B resistance and survival of
1147 *Leishmania donovani* under oxidative stress. *Redox Biology*. 2017 Aug;12:350–66.
- 1148 144. Collett CF, Kitson C, Baker N, Steele-Stallard HB, Santrot M-V, Hutchinson S, et al. Chemogenomic
1149 profiling of anti-leishmanial efficacy and resistance in the related kinetoplastid parasite *Trypanosoma*
1150 *brucei*. *Antimicrobial Agents and Chemotherapy*. 2019;(June).
- 1151 145. Armitage EG, Alqaisi AQI, Godzien J, Peña I, Mbekeani AJ, Alonso-Herranz V, et al. Complex interplay
1152 between sphingolipid and sterol metabolism revealed by perturbations to the leishmania metabolome
1153 caused by miltefosine. *Antimicrobial Agents and Chemotherapy*. 2018;62(5):1–12.
- 1154 146. Baldrige RD, Graham TR. Identification of residues defining phospholipid flippase substrate specificity
1155 of type IV P-type ATPases. *Proceedings of the National Academy of Sciences of the United States of*
1156 *America*. 2012;109(6).

- 1157 147. Casulli A. New global targets for NTDs in the WHO roadmap 2021-2030. 2021;
- 1158 148. Ekram MR, Amin MR, Hasan MJ, Khan MAS, Nath R, Mallik PK, et al. Efficacy and safety of single-
1159 dose liposomal amphotericin B in patients with visceral leishmaniasis in Bangladesh: a real-life
1160 experience. *Journal of Parasitic Diseases*. 2021;
- 1161 149. Merlen T, Sereno D, Brajon N, Rostand F. *Leishmania* spp; : completely defined medium without serum
1162 and macromolecules (CDM/LP) for the continuous in vitro cultivation of infective promastigote forms.
1163 Vol. 60, *Ain. J. Trop Med. Hyg.* 1999.
- 1164 150. Nayak A, Akpunarlieva S, Barrett M, Burchmore R. A defined medium for *Leishmania* culture allows
1165 definition of essential amino acids. *Experimental Parasitology*. 2018;185:39–52.
- 1166 151. Bates PA, Tetley L. *Leishmania mexicana*: Induction of Metacyclogenesis by Cultivation of
1167 Promastigotes at Acidic pH. *Experimental Parasitology*. 1993 Jun 1;76(4):412–23.
- 1168 152. Vermeersch M, Da Luz RI, Toté K, Timmermans JP, Cos P, Maes L. In vitro susceptibilities of
1169 *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: Practical
1170 relevance of stage-specific differences. *Antimicrobial Agents and Chemotherapy*. 2009;53(9):3855–9.
- 1171 153. Bates PA, Robertson CD, Coombs GH, Tetley L. Axenic cultivation and characterization of *Leishmania*
1172 *mexicana* amastigote-like forms. *Parasitology*. 1992 Oct 6;105(2):193–202.
- 1173 154. Shimony O, Jaffe CL. Rapid fluorescent assay for screening drugs on *Leishmania* amastigotes. *Journal of*
1174 *Microbiological Methods*. 2008;75(2):196–200.
- 1175 155. Mikus J, Steverding D. A simple colorimetric method to screen drug cytotoxicity against *Leishmania*
1176 using the dye Alamar Blue®. *Parasitology International*. 2000;48(3):265–9.
- 1177 156. Pan AA. *Leishmania mexicana*: Serial cultivation of intracellular stages in a cell-free medium.
1178 *Experimental Parasitology*. 1984;58(1):72–80.
- 1179 157. Díaz M V., Miranda MR, Campos-Estrada C, Reigada C, Maya JD, Pereira CA, et al. Pentamidine exerts
1180 in vitro and in vivo anti *Trypanosoma cruzi* activity and inhibits the polyamine transport in *Trypanosoma*
1181 *cruzi*. *Acta Tropica*. 2014;134(1):1–9.
- 1182 158. Shrivastava R, Tupperwar N, Drory-Retwitzer M, Shapira M. Deletion of a Single LeishIF4E-3 Allele by
1183 the CRISPR-Cas9 System Alters Cell Morphology and Infectivity of *Leishmania*. 2019;
- 1184 159. Maes L, Beyers J, Mondelaers A, Van den Kerkhof M, Eberhardt E, Caljon G, et al. *In vitro* ‘time-to-kill’
1185 assay to assess the cidal activity dynamics of current reference drugs against *Leishmania donovani* and
1186 *Leishmania infantum*. *Journal of Antimicrobial Chemotherapy*. 2017 Feb 1;72(2):428–30.
- 1187 160. Ismael AY, Garmson JC, Molyneux DH, Bates PA. Transformation, development, and transmission of
1188 axenically cultured amastigotes of *Leishmania mexicana* in vitro and in *Lutzomyia longipalpis*. *American*
1189 *Journal of Tropical Medicine and Hygiene*. 1998;
- 1190 161. Scheltema RA, Jankevics A, Jansen RC, Swertz MA, Breitling R. PeakML/mzMatch: A File Format, Java
1191 Library, R Library, and Tool-Chain for Mass Spectrometry Data Analysis. *Analytical Chemistry*. 2011
1192 Apr;83(7):2786–93.
- 1193 162. Creek DJ, Jankevics A, Burgess KE V., Breitling R, Barrett MP. IDEOM: an Excel interface for analysis
1194 of LC–MS-based metabolomics data. *Bioinformatics*. 2012 Apr 1;28(7):1048–9.
- 1195 163. Creek DJ, Anderson J, McConville MJ, Barrett MP. Metabolomic analysis of trypanosomatid protozoa.
1196 *Molecular and Biochemical Parasitology*. 2012 Feb;181(2):73–84.
- 1197 164. Srivastava Anubhav and Creek DJ. Using the IDEOM Workflow for LCMS-Based Metabolomics Studies
1198 of Drug Mechanisms. In: Li S, editor. *Computational Methods and Data Analysis for Metabolomics*. New
1199 York, NY: Springer US; 2020. p. 419–45.
- 1200 165. Haug K, Cochrane K, Nainala VC, Williams M, Chang J, Jayaseelan KV, et al. MetaboLights: A resource
1201 evolving in response to the needs of its scientific community. *Nucleic Acids Research*. 2020 Jan
1202 1;48(D1):D440–4.

166. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. 2013;00(00):1–3.
167. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome research*. 2010 Sep;20(9):1297–303.
168. Benjamin D, Sato T, Cibulskis K, Getz G, Stewart C, Lichtenstein L. Calling Somatic SNVs and Indels with Mutect2. 2013;
169. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly*. 2012;6(2):80–92.
170. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative Genome Viewer. *Nature Biotechnology*. 2011;29(1):24–6.
171. Thompson J, Higgins D, Gibson T. CLUSTAL W: improving the sensitivity of progressive position-specific gap penalties and weight matrix choice multiple sequence alignment through sequence weighting. 1994 Sep;22(22):4673–80.
172. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al. STRING v10: Protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*. 2015;43(D1):D447–52.
173. Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, et al. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Research*. 2011 Jan 1;39(Database):D561–8.
174. Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, et al. STRING 8 - A global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Research*. 2009;37(SUPPL. 1):412–6.
175. von Mering C, Jensen LJ, Snel B, Hooper SD, Krupp M, Foglierini M, et al. STRING: Known and predicted protein-protein associations, integrated and transferred across organisms. *Nucleic Acids Research*. 2005;33(DATABASE ISS.):433–7.

Supplementary Information

S1 Table. List of polyene resistant lines selected for downstream analyses.

A) List of the most resistant clones chosen from each line. **B)** List of individual clones selected for downstream analyses.

(XLS).

S2 Table. Formulation of HOMEM and DM culture media.

A) HOMEM. **B)** Defined Medium (DM). Medium supplemented with serum and antibiotics as described in Material and Methods.

(XLS).

S3 Table. Drugs susceptibility profile (EC50 values) in all polyene resistant lines of *Leishmania* spp.

Mean EC₅₀ values (μM) ± Standard Deviation (SD). Except for NMC tested in duplicates, values are from at least three biological replicates. One-way ANOVA was performed independently for each compound to determine differences of the mean between groups. Tukey's test compared pairwise differences of resistant lines with respect the parental wild type. Statistical difference (P<0.05, 95% Confidence Interval) is shown with stars as follows: ns non-significant or P>0.05; * P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001.). ND: not determined. AmB (amphotericin B), NMC (natamycin), Nys (nystatin), keto (ketoconazole), MF (miltefosine),

- 1247 PAR (paromomycin), PAT (antimony potassium tartrate), PENT (pentamidine), MB (methylene blue). See
1248 Material and Methods for full details.
- 1249 (XLS).
- 1250 **S4 Table. LC-MS data of AmBR lines cultured in HOMEM and DM.**
- 1251 Metabolites in the pentose-phosphate pathway detected with LC-MS in mid log wild type parasites (1 x 10⁸)
1252 cultured in HOMEM or DM and treated with high concentrations of AmB for 15 mins (See Material and
1253 Methods for a full description). Multivariate data analysis was performed with PiMP analysis pipeline (118) and
1254 the Benjamini-Hochberg procedure adjusted raw P-values (q-values) < 0.05 for ANOVA. Each experiment
1255 represents one biological replicate (n=4).
- 1256 (XLS).
- 1257 **S5 Table. Sterol peaks identification and composition (%) found by GC-MS in polyene (AmB and Nys)**
1258 **resistant lines of *Leishmania* spp.**
- 1259 **A)** Identification of sterol peaks by GC-MS. Standards used were: cholesterol, TMS; desmosterol, TMS; 5 α -
1260 cholest-7-en-3 β -ol, TMS; ergosterol, TMS; stigmasterol, TMS; β -sitosterol, TMS; lanosterol, TMS; FF-MAS
1261 (4,4-dimethyl-5 α -cholesta-8,14,24-trien-3 β -ol) and zymosterol. FF-MAS: Follicular fluid meiosis-activating
1262 sterol and an intermediate in the cholesterol biosynthetic pathway present in all cells. Peaks that did not match
1263 any standard were determined by comparing with the NIST spectral libraries with the ion trap mass
1264 spectrometer. 5-dehydroepisterol based on literature (de Souza et al. 2009; Xu et al. 2014; Yao et al. 2013; Yao
1265 and Wilson 2016). RT: retention time. TMS: trimethylsilyl (for derivatization). **B)** Sterol composition (%) by
1266 GC-MS in polyene (AmB and Nys) resistant lines of *Leishmania* spp. promastigotes and amastigotes. Content
1267 of each sterol is the percentage of the total of the raw peak area detected per line $\pm$ Standard deviation of three
1268 independent biological replicates. Sterol content is shown as the total (%) of the raw peak area detected per
1269 sample ($\pm$ SD) from all biological replicates (n=3).
- 1270 (XLS).
- 1271 **S6 Table. Genotype of mutation identified in sterol genes in polyene resistant and parental wild type of**
1272 ***Leishmania* spp.**
- 1273 LOX (C5DS): Lathosterol oxidase a.k.a. C-5 desaturase (*LmxM.23.1300*); * The genotype in both C24SMT
1274 copies is G961/A961 for *LmxM.36.2380* & *LmxM.36.2390* (*LINF_360031200* & *LINF_360031300* in *L.*
1275 *infantum*), respectively. ‡ these mutants showed low or no coverage of the intergenic region between both
1276 copies of C24SMT (S6 Fig). ND= Non determined due to total absence of coverage at this locus. Changes in the
1277 miltefosine transporter (MT) *LmxM.13.1530* are included here and the visualization of genome coverage at this
1278 locus is shown in S7 Fig.
- 1279 (XLS).
- 1280 **S7 Table. Susceptibility to AmB of AmBR *L. mexicana* axenic promastigotes before and after infection in**
1281 **mice and in infected macrophages.**
- 1282 Values in μ M, Mean $\pm$ Standard Deviation (SD). One-way ANOVA was performed independently for each
1283 compound to determine differences of the mean between groups. Tukey's multiple compared pairwise
1284 differences of resistant lines with respect the parental wild type. Statistical difference (P<0.05, 95% Confidence
1285 Interval) is shown with stars as follows: ns non-significant or P>0.05; * P $\leq$ 0.05; ** P $\leq$ 0.01; *** P $\leq$ 0.001; ****
1286 P $\leq$ 0.0001. FC: Fold Change of AmBR lines is relative to their respective parental wild type.
- 1287 (XLS).
- 1288 **S8 Table. Copy Number Variation (CNV) in all polyene resistant lines of *Leishmania* spp.**
- 1289 Copy number ratio was obtained by comparing the resistant lines with the corresponding wild type line which is
1290 used as the base line. Gene ID (column A-I) were obtained from TriTrypDB-46. Reference genomes were
1291 divided into equally sized bins of 1000 base pairs and the start and end points of the called segments were
1292 approximate and not accurate (see Material and Methods for a full description). A visualization of this Table is
1293 shown in S8 Fig.

1294 (XLS).

1295 **S9 Table. Protein altering mutations (SNPs) identified in all polyene resistant lines of *Leishmania* spp.**

1296 Paired end reads were aligned with the corresponding wild type parental line. Reference genome and gene IDs
1297 were retrieved from TriTrypDB database (see Material and Methods for a full description). The most relevant
1298 mutations are listed in Table 1.

1299 (XLS).

1300 **S1 Fig. Selection pressure in polyene-resistant lines of *Leishmania* spp. promastigotes.**

1301 *L. mexicana* (panels A to D) and *L. infantum* (panel E) mid-log promastigotes (5×10^5) were grown with a
1302 stepwise increasing concentration of AmB (panels A, C-E) or Nys (panel B) added in the cultured medium and
1303 indicated with coloured pipelines (left y-axis). The EC₅₀ (right-y axis) is shown for wild type (black bars) and
1304 resistant lines, AmBR and NysR (coloured bars in all panels). The mean EC₅₀ of the parental wild type is shown
1305 (horizontal dotted lines). See Material and Methods for a detailed description.

1306 (PNG).

1307 **S2 Fig. Growth rate and size of polyene resistant *L. mexicana* promastigotes.**

1308 Cell density was measured every 24 hours for 8 days starting from 1×10^5 cells/ml. **A)** AmBR lines in
1309 HOMEM. **B)** NysR in HOMEM. **C)** AmBRA4 in HOMEM and AmBRA3-DM in Defined Medium (DM). **D)**
1310 Growth kinetics of AmBR lines from panel A (HOMEM) cultured in DM. **E)** Violin plot of the mean size (μ m)
1311 of four AmBR lines from panel A. The central continuous line within each coloured violin plot is the median of
1312 each group. The cell body length was measured from the base of the flagellum until the posterior endpoint of the
1313 cell body of promastigotes in the stationary phase. Data were processed with ImageJ software and represent the
1314 mean of the sample ($n \geq 30$). Measurements are the mean ($\pm$ SD) of three biological replicates. Tukey's multiple
1315 comparison test was used to find pairwise differences between resistant lines and parental wild type. Statistically
1316 significant values ($P < 0.05$, 95% Confidence Interval) are indicated with stars as follows: * $P \leq 0.05$, ** $P \leq 0.01$,
1317 *** $P \leq 0.001$, **** $P \leq 0.0001$.

1318 (PNG).

1319 **S3 Fig. Fold change in the EC₅₀ of antileishmanials in all polyene resistant lines of *Leishmania* spp.**

1320 Fold change in EC₅₀ is relative to the parental to the respective parental wild type cultured in parallel without
1321 drug. Values higher and lower than 1, indicate a decrease and increase in susceptibility, respectively. AmB
1322 (amphotericin B), NMC (natamycin), Nys (nystatin), keto (ketoconazole), MF (miltefosine), PAR
1323 (paromomycin), PAT (Antimony potassium tartrate), PENT (pentamidine) and MB (Methylene blue).

1324 (PDF).

1325 **S4 Fig. Visualization of the genomic region in the sterol gene *LmxM.23.1300* in AmBRcl.3.**

1326 The image shows two independent heterozygous mutations, G220A (V74M) and T221A (V74E) in AmBRcl.3.
1327 The red square indicates the localisation of both mutation in different reads. Image was produced with WGS
1328 data aligned to the reference genome (<https://tritrypdb.org>) using the IGV_2.8.9 software
1329 (<http://software.broadinstitute.org/software/igv/>). See Fig 5 for a full description of the panels.

1330 (PNG).

1331 **S5 Fig. Alignment of the *Leishmania* gene lathosterol oxidase (or sterol C5-desaturase).**

1332 *L. mexicana* gene *LmxM.23.1300* (LOX). Black boxes and stars denote His and other residues conserved across
1333 species, respectively. Novel variants identified in this study are indicated with black (AmBR lines) and red
1334 (NysR line) arrows. Also included (blue box) is the variant from our previous study (Pountain et al. 2019). From
1335 top to bottom kinetoplasts listed are *L. mexicana*, *L. major*, *L. infantum*, *L. braziliensis*, *T. brucei*, *T. cruzi*,
1336 *Bodo saltans* and *Crithidia fasciculata*. Also included are *D. rerio* (zebra fish), *M. musculus* (mouse), *Homo*
1337 *sapiens* (human), *S. cerevisiae* (budding yeast), *C. albicans* (pathogenic fungi) and *Arabidopsis thaliana* (plant).

Proteins sequences are from TriTrypDB (<https://tritrypdb.org>) and Uniprot (<https://www.uniprot.org/>). Alignment was performed using Clustal Ω with default settings (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

(PNG).

S6 Fig. Coverage of *LmxM.36.2380* and *LmxM.36.2390* in polyene resistant lines cultured in HOMEM and DM.

A-B) AmBR lines (and their respective parental WT) cultured in HOMEM showing the absence of coverage in this locus in five lines. **C)** AmBR lines and wild type cultured in DM. **D)** Reads coverage in the intergenic region in NysRcl.B2 and its parental WT. **E)** *L. infantum* showing total absence (AmBRcl.G5) and partial coverage (parental WT). Reads were aligned to the reference genome (<https://tritrypdb.org>) and the image was produced using the software IGV 2.8.9 (<http://software.broadinstitute.org/software/igv/>). See Fig 5 for a full description of the panels.

(PNG).

S7 Fig. Coverage of the miltefosine transporter of (MT) and adjacent genes in *Leishmania* spp.

The coordinates of a region spanning a genomic region of ~16 kb and ~26 kb are shown. Genes are shown at the bottom (blue bars). **A-B)** coverage in NysRcl.B2 and AmBRcl.G5 **C)** In AmBRcl.8 and AmBRcl.6 a total absence of coverage of a region (~9 kb) comprising *LmxM.13.1530* and adjacent gene downstream *LmxM.13.1540* is shown while small gaps of low coverage are observed downstream from each gene in AmBcl.14, AmBcl.3 and WT. **D)** Absence of a ~20 kb region comprising four genes (from *LmxM.13.1510* to *LmxM.13.1540*) is shown in AmBRA4. WGS data were aligned to the reference genome (<https://tritrypdb.org>) and the images were produced with IGV_2.8.9 software (<http://software.broadinstitute.org/software/igv/>). See Fig 5 for a full description of the panels.

(PNG).

S8 Fig. Copy Number Variation (CNV) in all polyene resistant lines of *Leishmania* spp.

Copy ratio alterations were detected using GATK (version 4.2.0.0). Reference genomes from TriTrypDB46 (<https://tritrypdb.org>) were divided into equally sized bins of 1000 base pairs (start and end points of the called segments were approximate). CN ratio is obtained by comparing the resistant lines with the corresponding wild type line. Plots of denoised and segmented copy-ratios were generated using R. The thick black lines show the mean copy ratios of corresponding segments. Values of this Fig are provided in S8 Table.

(XLS).

S9 Fig. Visualization of the variants in glutathionylspermidine synthase (GSS).

A) Genomic region showing a heterozygous mutation (boxed) in GSS in AmBRcl.G5 of *L. infantum*. **B)** Protein-protein interaction (PPIs) map of GSS and other enzymes relevant for the trypanothione biosynthesis. GSS (LINF_250031200), GSS: glutathione synthetase (LINF_140015200), TRYS: trypanothione synthetase (LINF_270025600), glutathione peroxidase (LINF_360038100), trypanothione synthetase (LINF_230009800), spermidine synthase (LINF_040010800), trypanosome basal body component (LINF_240028000) and two mannosyltransferases (LINF_360027400 and LINF_120006700). **C)** Genomic region showing the heterozygous mutation in GSS in two lines, AmBRcl.8 and AmBRcl.6 of *L. mexicana*. The reference genome and gene IDs were retrieved from TriTrypDB (<https://tritrypdb.org>). Images were produced using IGV 2.8.9 (<http://software.broadinstitute.org>) and STRING (<https://string-db.org>). See Fig 5 for a full description of the panels A and C.

(PNG).

S10 Fig. Summary of mutations in all polyene resistant lines of *Leishmania* spp. (List of genes).

Gene ID shown on the left were retrieved from TriTrypDB. All polyene resistant lines are shown in the x-axis and the mutation(s) type present in each are coloured coded. This image was produced with R package. See S9 Table for a full list of these variants.

1383 **S11 Fig. Histopathology of primary lesions (footpad) of BALB/c mice inoculated with AmBR lines of *L.***
1384 ***mexicana*.**

1385 **A1-2, B1-2)** AmBRcl.14 (yellow) and AmBRcl.3 (orange) infection with intense parasitic load and
1386 inflammatory infiltrate within skin histiocytes. **C1-2)** *L. mexicana* wild type (green) causing diffuse
1387 inflammatory infiltration. Internalised amastigotes are indicated (black arrows). **D1-2, E1-2)** infection with
1388 AmBRcl.8 and AmBRcl.6 (dark and light blue) and discrete inflammatory reaction localised in papillary dermis
1389 without visible parasites. Samples are representative of three biological replicates. Hematoxylin-eosin (H&E)
1390 stain. Scale bar ~10 μ M Objective ~63x.

1391 (PNG).

1392 **S12 Fig. Retention of resistance (EC50 values) to AmB in *L. mexicana*-AmBR lines recovered from mice**
1393 **tissue (footpad and lymph nodes) after treatment with AmB (1 mg/kg).**

1394 Susceptibility to AmB in AmBR *L. mexicana* axenic promastigotes after infection in mice. Parasites were
1395 recovered as amastigotes from mice tissue (lymph nodes and footpad) and transformed into promastigotes in
1396 HOMEM culture medium. Mice were treated with AmB (1 mg/kg) at week thirteen or left untreated (control
1397 group). See Material and Methods for a full description.

1398 (PNG).

Fig 1A

AmBR lines (*L. mexicana*) in HOMEM

Fig 1B

NysR lines (*L. mexicana*) in HOMEM

Fig 1C

AmBR lines (*L. mexicana*) in DM

Fig 1D

AmBRcl.G5 (*L. infantum*) in HOMEM

Fig 1 E

		Culture Media							
		HOMEM						DM	
PPP Metabolites	Formula	LmWT1	AmBRcl.14	LmWT1	AmBRcl.8	LmWT2	AmBRB2	LmWT-DM	AmBRA3-DM
Sedoheptulose 7-phosphate	C7H15O10P	2.69	3.98	-0.36	-0.18	0.81	0.07	-1.07	-3.18
Ribose 5-phosphate	C5H11O8P	2.75	2.66	3.15	2.38	1.47	1.89		
Glucose 6-phosphate	C6H13O9P	1.68	1.61	2.39	6.30	0.61	1.17	-0.37	-2.92
Sedoheptulose 7-phosphate	C7H15O10P	0.41	0.24	2.91	2.37	1.06	1.53	-0.22	-3.96
5-Phospho-alpha-D-ribose 1-diphosphate	C5H13O14P3	-4.86	0.00						
Glucono-1,5-lactone	C6H10O6	0.10	-0.08					-2.63	-3.11
Erythrose 4-phosphate	C4H9O7P	-1.06	-0.28					-2.08	-4.22
Pyruvic acid	C3H4O3	-1.48	-0.67			0.69	0.54		
Glyceraldehyde	C3H6O3	-0.84	-0.69	0.66	1.03	0.03	0.24	-0.54	-1.49
Glyceraldehyde 3-phosphate	C3H7O6P	-1.90	-0.86			1.21	1.10	-1.16	-1.59
2-Keto-D-gluconic acid	C6H10O7	-0.64	-0.86						
6-Phospho-D-gluconate	C6H13O10P	-0.33	-1.16	5.88	2.02	2.03	2.31	1.06	-1.39
2-Phospho-D-glycerate	C3H7O7P	-1.62	-2.10	0.53	-0.48	-0.17	0.48	0.14	-2.93
Fructose 1,6-bisphosphate	C6H14O12P2	-4.51	-2.77			0.82	1.48	0.57	-0.02
Glucose	C6H12O6	-3.34	-2.81	-0.73	-0.48	-0.30	-0.15	-0.59	-0.89
Ribose	C5H10O5	-5.15	-3.45			-0.04	0.14	-1.44	-3.85
Deoxyribose	C5H10O4	-6.94	-3.92	-1.87	-1.66	-0.71	-0.71	-2.65	-4.74
Ribose 1,5-bisphosphate	C5H12O11P2	-1.98	-4.29						
Glycerate	C3H6O4							2.50	-0.34
Glucono-1,5-lactone 6-phosphate	C6H11O9P			5.63		2.37	1.32		

Fig 2C

A) AmBR lines in HOMEM

B) AmBR lines in DM

C) AmBR lines (*L. infantum*) in HOMEM

D) NysR lines in HOMEM

E) AmBR lines in HOMEM (amastigotes)

Fig 4

Fig 5A

Fig 5B

MT (ID= *LmxM.13.1530*)

Fig 5C

Response to AmB in BALB/c mice infected AmBR lines of *L. mexicana*

Fig 6A

Fig 6B

Infected macrophages

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Fig 6C

HOMEM (AmBRcl.8)

Figure 2A

Figure 2B