1   **A complex interplay between sphingolipid and sterol metabolism revealed by**

2   **perturbations to the _Leishmania_ metabolome caused by miltefosine**

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20   **Running title: Miltefosine on sphingolipids and sterols in _Leishmania_**

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ABSTRACT

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26   With the World Health Organization reporting over 30,000 deaths and 200-400,000 new cases

27   annually, visceral *Leishmania*sis is a serious disease affecting some of the world's poorest people. As

28   drug resistance continues to rise, there is a huge unmet need to improve treatment. Miltefosine

29   remains one of the main treatments for *Leishmania*sis, yet its mode of action (MoA) is still unknown.

30   Understanding the MoA of this drug and parasite response to treatment could help pave the way for

31   new, more successful treatments for *Leishmania*sis. A novel method has been devised to study the

32   metabolome and lipidome of *Leishmania donovani* axenic amastigotes treated with miltefosine.

33   Miltefosine caused a dramatic decrease in many membrane phospholipids (PLs), in addition to

34   amino acid pools, while sphingolipids (SLs) and sterols increased. *Leishmania major* promastigotes

35   devoid of SL biosynthesis through loss of the serine palmitoyl transferase gene (ΔLCB2) were 3-fold

36   less sensitive to miltefosine than WT parasites. Changes in the metabolome and lipidome of

37   miltefosine treated *L. major* mirrored those of *L. donovani*. A lack of SLs in the ΔLCB2 was matched

38   by substantial alterations in sterol content. Together these data indicate that SLs and ergosterol are

39   important for miltefosine sensitivity and perhaps, MoA.

40

INTRODUCTION

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42   Infectious diseases continue to cause great morbidity and mortality worldwide(1). New drugs are

43   required and will need to be continuously replenished as resistance to antimicrobials increases.

44   Understanding the mode of action (MoA) of currently available treatments against microbial

45   diseases offers a means to highlight targets for new treatments. Metabolomics plays an important

46   role in this discovery and development of new medicines for infectious diseases(1).

47   The *Leishmania*ses are a spectrum of neglected tropical diseases caused by protozoa of the genus

48   *Leishmania.* Individual species provoke different clinical manifestations including visceral

49   *Leishmania*sis caused by *Leishmania donovani* and *Leishmania infantum*(2) which is fatal if not

50   treated. Existing therapeutic options are limited(3), so the search for alternative therapies continues.

51   Two key developmental stages of *Leishmania*: amastigotes and more commonly promastigotes are

52   used for *in vitro* studies of drug MoA. Promastigotes are the form found in the sand fly vector, while

53   amastigotes exist in the mammalian host. Development of axenic cultures, having physiological

54   similarity to the macrophage resident forms in mammalian infections, has made it possible to study

55   amastigotes *in vitro*(2).

56   Metabolomics seeks comprehensive measurements of small molecules in a given system. However,

57   the dynamic range in abundance and broad physicochemical diversity of metabolites is such that a

58   single analytical platform is lacking. Here, a combined liquid chromatography – mass spectrometry

59   (LC-MS) and capillary electrophoresis – mass spectrometry (CE-MS) approach was used to increase

60   coverage of the *Leishmania* metabolome and applied to study MoA of miltefosine, the first drug

61   approved for oral treatment of *Leishmania*sis. Metabolomics has proven useful in drug MoA studies

62   for *Leishmania* promastigotes(4–13), but so far not for *L. donovani* amastigotes. *L. mexicana*

63   amastigotes were recently studied using metabolomics to show that amastigote differentiation is

64   associated with the induction of a distinct stringent metabolic state(14), in both lesion-derived and

65   *in vitro* differentiated amastigotes.

66   Several suggestions have been made regarding the anti-leishmanial action of miltefosine: induction

67   of apoptosis-like death(15, 16), or disruption of metabolite transport(4, 17, 18). The uptake of

68   miltefosine in *Leishmania* is dependent on transmembrane lipid transporters, most notably the

69   flippase LdMT and its accessory protein – LdRos that are commonly lost with selection of

70   resistance(19). More recently, using cosmid-based functional cloning coupled to next-generation

71 sequencing, genes involved in ergosterol biosynthesis and phospholipid (PL) translocation were

72 suggested to contribute to resistance in *L. infantum*(20).

73 Metabolomic analyses of miltefosine treated *L. infantum* showed a general depletion of intracellular

74 metabolites(6) and similar studies in other *Leishmania* demonstrated lipid remodelling(21–24).

75 Biochemical modifications to different lipid classes have been reported in the membranes of

76 miltefosine treated *L .donovani* promastigotes(22). In addition to diminishing phosphatidylcholine

77 (PC), miltefosine was found to double sterol composition too(22). Effects of miltefosine treatment

78 on lipid metabolism in promastigotes of *L. infantum*(6) have also been observed using

79 metabolomics, although only lipid class rather than individual lipid species were resolved. Combining

80 CE-MS to analyse polar and ionic metabolites and reversed phase LC-MS to reveal specific lipidomic

81 changes in *L .donovani* has allowed a more detailed analysis into the MoA in axenic amastigotes

82 presented herein.

83 Substantial changes in sphingolipid (SL) and sterol metabolism were revealed in miltefosine treated

84 *L. donovani* promastigotes. SLs were first reported in *L. donovani* more than 20 years ago(25) and

85 along with sterols, have since received attention in many species(26–28). *Leishmania* obtain SLs *via*

86 salvage or *de novo* synthesis(29), and they play important roles in differentiation, replication,

87 trafficking and virulence(29). The prominent, and most studied, SL identified is inositol

88 phosphoceramide (IPC), although *Leishmania* are believed to exhibit a complete and functional SL

89 pathway involving both biosynthesis and degradation(28, 30). A more comprehensive analysis of

90 different SLs may identify other key targets of SL metabolism for therapy. To study the viability of

91 *Leishmania* without SL synthesis, a mutant was created by deletion of the gene encoding the

92 essential catalytic subunit of the serine palmitoyltransferase (ΔLCB2), the first and rate-limiting step

93 in SL biosynthesis(28, 31). Surprisingly, this mutant was viable, indicating a dispensable role for SLs in

94 *Leishmania,* unlike in the related parasite *Trypanosoma brucei*(32). It was suggested that the sterol

95 composition of the *Leishmania* plasma membrane, where ergosterol replaces cholesterol as the

96   primary membrane sterol, could enable this(32). Here perturbations to *Leishmania* SL and sterol

97   metabolism on miltefosine treatment are described, the interplay between these metabolite

98   families is considered and the role sterols play in drug sensitivity proposed.

99   **RESULTS AND DISCUSSION**

100   In order to define the optimal protocol for sampling/quenching/extracting/analysing metabolites

101   from *L. donovani* axenic amastigotes, a method was developed as described below and further in the

102   Supplementary Information (SI - file 1). The extraction procedure was optimised such that LC-MS and

103   CE-MS analyses could be performed from single samples of as few as 1×10^7 parasites (SI Figure 1S:).

104   For *L. donovani*, samples were treated with 4.47 µM (the observed EC_{50} at 72 hours, consistent with

105   the literature (33)) or 13.41 µM (three times the observed EC_{50} at 72 hours) miltefosine and

106   harvested after 5h or 24h of exposure to observe the initial effects of the drug. For *L. major,* samples

107   were treated with 10 µM or 30 µM miltefosine and harvested after 5h of exposure. DMSO controls

108   were prepared alongside treated samples at each time-point for both species. Results from method

109   development stages are detailed in the SI, where a comparison of methanol extraction and a more

110   comprehensive extraction for lipidomics using LC-MS is shown in figure 2S, profiles obtained using

111   LC-MS and CE-MS analysis of different extractions are shown in figure 3S and the final optimised

112   dual extraction procedure to obtain different extracts for LC-MS and CE-MS analysis from single

113   samples is shown in figure 4S.

114   Metabolomic determination of *Leishmania* response to miltefosine

115   With the exception of the aforementioned work on *L. mexicana* amastigotes(14), the few studies on

116   drug MoA in *Leishmania* have focussed on the promastigote form(2–10). The aim of this research

117   was to explore effects on metabolism of miltefosine in *L. donovani* axenic amastigotes and, as a

118   result of these findings, in *L. major* promastigotes.

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119   After verification of quality (SI Figure 5S), data were divided into separate sets and differences

120   between treated and untreated parasites identified. Around 20 metabolites, including the drug

121   itself, were detected only in treated samples. These features, listed after the supplementary tables

122   in supplementary information, were all found to elute with the drug and therefore most likely

123   enhanced by the ionisation of the drug. These could not be identified as endogenous lipids and were

124   therefore assumed to be mass spectrometry derivatives of the drug itself. All were removed prior to

125   multivariate analysis to avoid separation based on presence or absence of drug alone. Identification

126   was performed for metabolite features found to increase/decrease with treatment after 5 or 24 h of

127   exposure, determined by a p-value <0.05 (Student's two-tailed t-test, n=6 per group) and a fold

128   change of ±1.5, calculated for at least one of the comparisons made. Identification of metabolites

129   found by CE-MS was confirmed by injection of authentic standards (as detailed in Supplementary

130   Table 1). Lipids detected using LC-MS were annotated considering chemical properties and elution

131   order. Miltefosine treatment affected different metabolic classes and possible impact on MoA is

132   discussed below, based on data presented in the Supplementary Tables (STs), a description for which

133   is given in SI.

134   _L. donovani_ axenic amastigotes

135   Miltefosine has been proposed to affect the transport of different metabolites(4, 17, 18). Consistent

136   with previous literature(6), miltefosine induced decreases in the majority of internal metabolites

137   detected by CE-MS, as shown in Table 1, which could be associated with impaired uptake. Figure 1

138   shows the abundances of metabolites associated with arginine metabolism detected in this study.

139   Arginine is a precursor to polyamine biosynthesis and a protein building block. Its intracellular

140   concentration is controlled by dedicated sensory protein transporters(10, 18), which have been

141   suggested as targets of miltefosine(4). Arginase, which catalyses the hydrolysis of arginine to

142   ornithine, also contribute to the intracellular concentration of arginine. The co-ordinated decrease in

143   arginine and ornithine may indicate a reduction in precursor levels (through blocking arginine

144 transport), a notion consistent with previously reported literature (34, 35). Another metabolite that

145 shares the same mass but has a distinct migration time to citrulline was identified as argininic acid

146 which is considered an endpoint of arginine metabolism previously found in different *Leishmania*

147 species, including *L. donovani*(36). Its concentration was substantially reduced with treatment at

148 both time-points and doses (up to 3-fold) in this research.

149 While data from CE-MS largely confirmed observations in the literature, the approach here has given

150 a finer-grained view of the effect of miltefosine on lipids. Compared to mammals, *Leishmania*

151 membrane lipids differ substantially in composition and function, making them important for

152 viability and virulence as well as potential drug targets(29). Lipids have been analysed previously in *L.*

153 *donovani*(7, 22, 29, 37), as have changes in lipid metabolism connected to miltefosine treatment(21–

154 23, 38, 39). Here we demonstrate more detail on individual lipid species than has previously been

155 reported.

156 In *L. infantum*, miltefosine was reported to alter 10 % of the metabolome, purportedly due to

157 compromised outer membrane integrity leading to lysis(6). Here, a general decrease in membrane

158 PL abundance was observed in *L.donovani* axenic amastigotes (see STs 2, 3 and 4 for specific lipids).

159 Miltefosine was reported by Zufferey *et al.* (2002) to inhibit PC biosynthesis, diminishing levels in *L.*

160 *donovani* promastigotes leading to growth arrest(21). Phospholipase D activity was unaffected by

161 the drug, hence inhibition of the choline transporter was proposed to underlie the reduction in PC

162 biosynthesis(21) . A number of PCs and other PLs were found diminished in this study too (ST2).

163 Other considerable effects of treatment observed were increases in sterols and, to an even greater

164 extent, SLs. To investigate this further, all filtered LC-MS data were scanned to identify peaks

165 identifiable as SLs (even if the relative levels in treated and untreated parasites were not statistically

166 different). Figure 2 shows the trends observed in SLs, where data are plotted for un-treated

167 parasites and parasites treated with the lowest dose of miltefosine at 24 h. A dramatic and

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168   significant (5-fold) increase in sphingosine abundance induced by miltefosine ($p=2\times10^{-6}$) was

169   mirrored by a 3-fold increase in sphinganine ($p=7\times10^{-4}$). All detected ceramides were also

170   substantially increased by 24 h, as shown and detailed in Supplementary Table 3.

171   *L. major* promastigotes

172   *Leishmania* SL metabolism has been best studied in the promastigote form of *L. major*, where a

173   ΔLCB2 mutant lacking the first enzyme of the biosynthetic pathway, serine palmitoyl transferase, is

174   available(28, 31).   The effects of miltefosine in these *L. major* promastigotes were therefore

175   investigated. The efficacy of miltefosine was established against both the ΔLCB2 mutant and wild

176   type lines; the EC_{50} for the wild type was 6.83 µM, consistent with previous literature (33), whilst for

177   mutant was three times higher at 21.21 µM. The lipidome of these parasites revealed major

178   differences between the wild type and ΔLCB2 lines and the effect of miltefosine was also compared.

179   Lipids identified with marked differences in abundance between any experimental groups compared

180   are detailed in STs 5, 6 and 7. As in *L. donovani,* miltefosine itself and around 20 other features were

181   detected only in treated samples. Miltefosine was identified in both wild type and ΔLCB2 mutant,

182   with no significant difference in relative concentration (p=0.09 for the lower dose and p=0.45 at the

183   higher dose). This demonstrated that the 3-fold resistance of the mutant was not due to inhibited

184   import. As in *L. donovani* in this study and reported in *L. infantum* previously(6), miltefosine caused

185   substantial effects in levels of numerous PLs in *L.major* too. This reduction may be due to reduced

186   import of PLs or choline, or reduced *de novo* biosynthesis.

187   Miltefosine's effects on *L. major* SLs were similar to those observed after 24h of exposure in *L.*

188   *donovani* amastigotes. Figure 3 shows the fold changes for both doses. As can be seen, some SLs

189   were detected only in *L .donovani* or only in *L .major*. This may be due to species specific differences

190   and could even be due to differences in the mechanism of each species given that they cause

191   different forms of leishmaniasis (*L. donovani* causing the visceral form and *L. major* the cutaneous

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192   form). The *L. major* ΔLCB2 mutants lack most SLs, in accordance with them being devoid of SL

193   biosynthesis. Sphingosine and ceramide (d36:1) were, however, detected indicating that they are

194   acquired from the media. Likewise, SM may also be derived from the media since there is no

195   evidence that *Leishmania* synthesize SM, although they do possess SMase which has been shown to

196   be essential in degrading host-derived SM to promote parasite survival, proliferation and

197   virulence(40). The increase in SLs could indicate a stimulatory effect of miltefosine on biosynthesis or

198   inhibition of a catabolic pathway, the latter seeming more likely since the mutant also accumulates

199   higher levels of the two SLs detected (SM and ceramide d36:1 upon treatment (Figure 3).

200   As in *L. donovani*, sterols were also increased by treatment in *L. major* wild type, though not in

201   ΔLCB2 mutants*.* The identification of sterols poses a particular challenge since many in the pathway

202   share identical masses. However, using the calculated LogP values it was possible to identify each

203   based on their elution order in the LC-MS data, as shown in SI file 2. Figure 4 shows the ergosterol

204   biosynthesis pathway and highlights observed increases in *L. donovani* after 5 h (blue arrows), 24 h

205   (red arrows) and in *L. major* wild type after 5 h (green arrows) of miltefosine exposure. Trends were

206   the same for both concentrations of the drug, except for fecosterol at the higher dose in *L. donovani*

207   which was increased albeit not significantly.

208   Although sterol differences were not observed with treatment in ΔLCB2 mutants (ST 7), comparison

209   to the un-treated wild type revealed dramatic differences in sterol metabolism, particularly with

210   respect to ergosterol and cholesterol. This alteration in sterol composition in the selection of the

211   mutant is particularly noteworthy since it enables the mutant to survive without SL synthesis, while

212   the change in sterol composition may stabilise membranes in the face of miltefosine treatment,

213   emphasising the complex interplay between SL and sterol metabolism. As shown in the

214   chromatographic peaks in Figure 4, ergosterol levels were significantly reduced (approximately

215   halved p=5 ×10^{-14}) in the ΔLCB2 relative to wild type whilst cholesterol levels were around 3-fold

216   more abundant p=8 ×10^{-12}. Cholesterol is probably scavenged in *Leishmania*(26). Ergosterol was

217   significantly increased with treatment in the wild type and of much lower abundance in the mutant

218   which exhibit a 3-fold reduced sensitivity to the drug. 5,7,22,24(28)-Ergostatetraenol, which

219   precedes ergosterol in its synthetic pathway, is also dramatically more abundant in ΔLCB2 mutants

220   compared to wild type, which suggests that the final step in ergosterol synthesis (catalysed by erg4)

221   is diminished in the mutant. Two further sterols, which share the same mass as ergosterol,

222   5,7,24(28)-Ergostatrienol and 5-dehydroepisterol, were observed in the mutant but were below the

223   limit of detection in the wild type. Their accumulation may also occur due to the reduced production

224   in ergosterol biosynthesis later in the pathway.

225   ΔLCB2 *Leishmania* mutants are viable while the same enzyme is essential to African trypanosomes.

226   This has been proposed to be due to *Leishmania* depending on ergosterol as its primary sterol

227   rather than cholesterol as in *T. brucei*(29)*.* However, since the ΔLCB2 mutant exhibits much higher

228   cholesterol and lower ergosterol concentrations relative to wild type, the simplistic view of changes

229   in cholesterol versus ergosterol appears to be inadequate to explain the essential nature of SL

230   synthesis in *T. brucei*. Though the magnitude of this sterol balance is not as severe as in *T. brucei*,

231   retained viability in the absence of SL synthesis is likely to be due to other reasons. As observed in

232   the *L.major* ΔLCB2 mutants, ergosterol reduction has been reported in a strain of *L. infantum*

233   resistant to 200 μM miltefosine compared to wild type(41), although the mechanism of resistance

234   was reported to be associated to mutations in the miltefosine transporter. In *L.donovani*

235   promastigotes, membrane sterol depletion has been correlated with reduced sensitivity to

236   miltefosine(42). In that study the authors tested a hypothesis that lipid rafts could be involved in

237   miltefosine action by destabilising these micro-domains through depletion of sterols using either

238   methyl-β-cyclodextrin (MCD) or cholesterol oxidase (CH-OX). Sterol depletion showed no significant

239   effects on the viability of either wild type or mutant, however MCD treatment significantly

240   decreased the susceptibility of wildtype to miltefosine (around 2-fold) although CH-OX depletion

241   caused no significant effect. Since MCD has less specificity in sterol extraction than CH-OX, MCD is

242   likely to deplete ergosterol and other sterols in addition to cholesterol, pointing to a possible link

243   between ergosterol depletion and reduced miltefosine activity. Increases in ergosterol in *L. donovani*

244   and *L. major* (wild type) co-ordinated with an increase in SLs reported here may also point towards a

245   function of lipid micro-domain complexes of sterols and SLs(27, 29, 42) in miltefosine's effects on

246   the parasites. Apoptosis has been proposed as an effect of miltefosine in tumour cells relating to

247   lipid micro-domains(43–45). Though the existence of apoptosis in *Leishmania* has been

248   challenged(46), various indications point to miltefosine inducing an apoptosis-like death in

249   *L. donovai* promastigotes(16). It is clear that miltefosine treatment causes profound changes to the

250   lipid content of *Leishmania* amastigotes and promastigotes and that alteration in lipid composition

251   such as a loss of SL biosynthesis and an accompanying change in sterol metabolism, impact on this

252   action of the drug.

253   **CONCLUSION**

254   A robust platform offering broad coverage of *Leishmania* metabolites using two complementary

255   techniques was developed to study miltefosine MoA. In addition to revealing miltefosine's effects

256   on internal metabolites and possible interference with membrane transport, many lipid species were

257   shown to be perturbed by treatment and importantly SLs and sterols were found to increase. These

258   findings, initially observed in *L. donovani* axenic amastigotes, were confirmed in *L. major*

259   promastigotes for which a defined ΔLCB2 mutant, devoid of SL biosynthesis was available. These

260   mutant parasites lacked SLs, other than those derived from the culture medium, and sterol

261   metabolism was drastically altered compared with wild type. This lipidomic remodelling was

262   associated with a 3-fold reduction in sensitivity to miltefosine. Coupled with the observation that

263   sterol concentrations increase when both *L. major* and *L. donovani* wild type parasites were exposed

264   to miltefosine, a major role for these lipids in miltefosine resistance and, perhaps, MoA is indicated.

265   **MATERIALS AND METHODS**

266   Experimental design

267   In order to define the optimal protocol for sampling/quenching/extracting/analysing metabolites

268   from *L. donovani* axenic amastigotes, a two-stage experiment was designed that i) allowed a

269   reproducible global profile of metabolites to be obtained and ii) allowed the execution of the

270   optimised protocol in the exploration of miltefosine MoA. It was necessary to optimise parasite

271   seeding densities and harvesting numbers to work with 5 h and 24 h time-points to obtain sufficient

272   biomass for metabolomics analyses, while also ensuring log phase of growth (metabolic steady state)

273   in parasites at the time of harvesting. Parasites were always seeded from log phase cultures to

274   minimise lag phase (particularly important at 5 h). Optimal seeding densities were determined as

275   6.67×10^6 parasites/mL for samples to be harvested at 5h and 1.33×10^6 parasites/mL for samples to

276   be harvested at 24h. Details of the method development are given in full in SI.

277   Chemicals and reagents

278   The axenic culture medium used in all experiments was prepared from one batch prepared 'in-

279   house' as described in Peña I. *et al*.(2). The culture medium used for *L. major* wild type and was

280   Schneider's Drosophila media (Sigma Aldrich) supplemented with heat inactivated foetal bovine sera

281   (15%). All methanol used was HPLC-grade and formic acid was analytical grade. These chemicals in

282   addition to formaldehyde solution and PBS were purchased from Sigma-Aldrich. Ultrapure water

283   was obtained using a Milli-Qplus 185 system (Millipore, Bilerica, MA, USA).

284   Sample collection and quenching of metabolism

285   *L. donovani* strain 1S2D (WHO designation: MHOM/SD/62/1S-CL2D)(47) was cultured by cycling

286   between promastigotes and axenic amastigotes using protocols from prior work(2). Briefly, the

287   promastigote form was grown at 29 °C and amastigote forms were grown at 37 °C with 5 % CO_2 in

288   different media adapted from De Rycker, *et al.*(48). For experiments with miltefosine, three T75

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289   flasks were prepared (with 30 mL culture at the appropriate densities for 5h or 24 h as described

290   above) for each condition: non-treated parasites, parasites treated with the lower dose of

291   miltefosine (4.47 µM) and parasites treated with the higher dose of miltefosine (13.41 µM).

292   At the time of sample harvesting, culture from each flask was divided equally into two 15 mL Falcon

293   tubes resulting in six replicate samples for each condition. Before the division, 50 µL of each culture

294   was collected into Eppendorf tubes to which 50 µL formaldehyde was added and samples were

295   stored at 4 °C to be counted later in order to record the exact number of parasites from each flask at

296   the time of harvesting. At the time of harvesting and throughout the subsequent processes, samples

297   were maintained at 4 °C.

298   After collection of culture, each sample was centrifuged at 1500 × g at 4 °C for 15 min after which

299   medium was decanted and parasites were washed in 2 mL PBS (maintained at 4 °C), then samples

300   were transferred to 2 mL Eppendorfs. From each sample, 10 µL was taken and fixed with 10 µL of

301   formaldehyde and stored at 4 °C to be counted later in order to record the exact number of

302   parasites in each sample immediately before quenching. Samples were subsequently centrifuged at

303   1500 × g at 4 °C for 15 min, PBS was decanted and 200 µL ice cold methanol was added to each

304   sample that were immediately stored at -80 °C until extraction and metabolomics analysis. Figure 1S

305   of Supplementary Information shows the workflow for the developed method for sampling.

306   *Leishmania major* parasites (MHOM/IL/81/Friedlin; FV1 strain) and a mutant in which the catalytic

307   subunit of serine palmitoyltransferase had been deleted by homologous recombination (ΔLCB2)(31)

308   were cultured as log phase promastigotes at 26°C. Samples were prepared for metabolomics as

309   described for *L. donovani* with the appropriate miltefosine doses as described above, except 10 µM

310   or 30 µM were used for the lower and higher dose of miltefosine respectively.

311   <u>Metabolite extraction</u>

312   Extraction blanks were prepared following all stages of extraction. On the day of analyses,

313   metabolites were extracted and supernatants analysed by LC-MS (and for all *L. donovani* samples in

314   CE-MS too). Samples were prepared by first evaporating extracts to dryness using a speed vacuum

315   concentrator (Eppendorf, Hamburg, Germany), after which 200 mg of 425-600 µm acid-washed glass

316   beads were added. Then to *L. donovani* samples, 575 µL of 100 % methanol were added, before

317   which samples were vortex mixed for 10 min and placed in a tissue lyzer for 30 min at 50 Hz. Finally

318   samples were centrifuged at 16,000 × *g* at 4 °C for 10 min and 80 µL of the resulting supernatants

319   were collected into LC-MS vials to be analysed directly. To the remaining samples (for CE-MS),

320   165 µL water were added and all vortex mixed for 30 min then centrifuged at 16,000 × *g* at 4 °C for

321   10 min, before being evaporated to dryness and re-suspended in 100 µL of water containing 0.2M

322   methionine sulfone (internal standard) and 0.1mM formic acid was added to each. QC samples for

323   LC-MS and CE-MS were prepared by collecting 10 µL from each sample into a single pool. For *L.*

324   *major*, only LC-MS extracts were prepared and therefore after evaporation, 120 mg of 425-600 µm

325   acid-washed glass beads and 350 µL methanol were added for extraction and 270 µL of the resulting

326   supernatants following extraction were collected into LC-MS vials to be analysed directly, from

327   which 30 µL was subtracted from each into a pool.

328   <u>Analysis of extracts by LC-MS and CE-MS</u>

329   For each analysis, extraction blanks were injected at the start of the analysis followed by eight

330   injections of the QC sample in order to ensure system stability, before the injection of samples

331   analysed in a random order with the QC injected after every sixth sample until the end of the

332   analysis. All instrumentation was from Agilent Technologies. For LC-MS, a 1290 infinity LC equipped

333   with reverse-phase column (Zorbax Extend C_{18} 50 × 2.1 mm, 3 µm; Agilent) was coupled to a 6550 Q-

334   TOF MS with electrospray ionisation source and operated in both positive and negative mode. For

335   CE-MS the instrument consisted of a 7100 CE coupled to a 6224 TOF MS operated in positive mode.

336   Details of the analytical procedures based on previously published methods(49, 50) are given in SI.

14

337   Data analysis and feature identification

338   Data from both platforms were processed using recursive analysis in Mass Hunter Profinder

339   (B.06.00, Agilent) software as detailed in SI. Data were reprocessed considering ions such as [M+H]$^+$

340   and [M+Na]$^+$, neutral water loss and the maximum permitted charge state was double. Alignment

341   was performed based on m/z and RT similarities within the samples. Parameters applied were 1 %

342   for the RT window and 20 ppm for mass tolerance.

343   Data treatment consisted of filtering based on quality, following the same procedure for each

344   dataset (*L. donovani*: LC-MS positive ion mode, LC-MS negative ion mode, CE-MS positive ion mode;

345   *L. major*: LC-MS positive ion mode, LC-MS negative ion mode). Data were filtered based on quality

346   using a quality assurance procedure described previously (QA+)(51). This involved retaining features

347   present in QCs at a rate of 80 % or absent in QCs (defined as presence < 20 %). For features present

348   in QC samples, only those with RSDs < 30 % were kept and for those absent, RSD was not calculated.

349   Then, for each comparison separately (5h, or 24h, wild type or ΔLCB2 mutant), features were further

350   filtered to keep only those present in at least five out of six of the replicates from one of the groups

351   simultaneously compared (resulting in a slightly different, but relevant dataset for each comparison).

352   For *L. donovani* multivariate analysis was employed firstly to observe the stability in each analysis

353   (LC-MS in positive and negative ionisation modes or CE-MS) as a whole and then for each time point

354   separately to investigate the effect of the drug on the parasite metabolome. To probe specific

355   questions on the effect of miltefosine at different time points or doses, fold-changes and *p*-values

356   were calculated in order to assess the degree of significance of any difference observed in the raw

357   data.

358   All significantly different metabolite features between un-treated and treated parasites at any dose,

359   determined by determined by a *p*-value <0.05 (Student's two-tailed *t*-test, *n*=6 per group) and a fold

360   change, ±1.5 were identified. Identification was performed searching *m/z* against Metlin

361 (http://metlin.scripps.edu) and lipidMAPS (http://lipidMAPS.org), considering the same adducts as

362 those described for data re-processing. Annotations were assigned to *m/z* values for metabolite

363 features considering i) mass accuracy (maximum mass error 10ppm); ii) isotopic pattern distribution;

364 iii) possibility of cation and anion formation and iv) adducts formation. This method of enhanced

365 annotation was based on our previously published work(49). Where possible, identifications were

366 compared by retention time order to standards analysed in-house. For CE-MS, definitive

367 identifications were made for a number of metabolites through analysis of authentic standards

368 analysed under the same conditions as the experiment, whereby samples were analysed again

369 followed by the same samples spiked with authentic standards to prove the identity.

370 All data analysed during this study are included in this published article and in the supplementary

371 tables (Supplementary Information files).

372 **ACKNOWLEDGEMENTS**

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537

538

539 **Table 1: Metabolites identified in *Leishmania donovani* axenic amastigotes and significantly affected by miltefosine.**

540

Identification (MSI level 1)	*m/z*	Migration Time	5 hours				24 hours			
			t-test		Fold change		*t*-test		Fold change	
			LD	HD	LD	HD	LD	HD	LD	HD
Acetyl-carnitine	204.1232	13.93	3.71E-02	2.30E-05	1.1	1.5	1.28E-03	4.37E-04	1.5	2.4
Adenosine	268.1042	15.23	NS	NS			8.03E-04	4.22E-04	3.7	57.3
Alanine	90.0552	13.95	6.86E-03	2.47E-03	1.3	1.4	2.49E-05	1.33E-06	1.7	5.4
Argininic acid	176.1032	14.19	1.32E-02	2.57E-04	1.2	1.6	9.30E-08	2.85E-08	2.8	6.8
Asparagine	133.0612	15.89	9.15E-03	2.60E-02	1.4	1.3	NS	4.30E-02		1.7
Betaine	118.0862	17.09	1.49E-02	NS	1.2		NS	4.51E-03		1.2
Choline	104.1072	10.92	1.35E-05	NS	1.5		3.24E-04	NS	1.4	
Citrulline	176.1032	16.68	9.08E-03	NS	1.3		1.47E-06	1.39E-05	1.6	1.4
Isoleucine	132.1022	15.52	2.56E-02	1.83E-04	1.3	3.0	1.39E-06	6.56E-06	2.1	4.2
Leucine	132.1022	15.65	4.61E-02	5.08E-03	1.3	1.8	3.91E-03	4.15E-04	1.9	3.2
Lysine	147.1122	11.14	5.50E-04	5.73E-05	1.6	2.7	1.29E-05	1.24E-06	2.1	2.9
Pipecolic acid	130.0862	15.62	6.08E-04	5.05E-04	1.8	1.8	3.78E-03	9.20E-07	1.3	4.3
Valine	118.0862	15.28	2.95E-03	5.84E-06	1.3	2.5	4.36E-06	3.20E-07	2.1	3.6
Glutamic acid	148.0682	16.51	NS	3.22E-05	1.1	1.3	4.18E-04	2.46E-09	1.2	4.7
Aspartic acid	134.0452	17.27	NS	NS			6.56E-03	1.64E-07	1.2	3.0
Creatine	132.0772	13.79	NS	NS			2.42E-04	1.21E-04	1.9	2.5
Histidine	156.0772	11.72	NS	2.49E-03		1.8	1.85E-06	1.55E-06	2.3	11.0
Arginine	175.1192	11.44	NS	9.87E-03		1.3	2.10E-08	1.18E-09	2.4	6.0
Proline	116.0702	11.04	NS	1.39E-02		2.1	5.35E-06	6.07E-06	4.0	40.2
Ornithine	133.0972	11.06	NS	1.45E-02		2.0	7.53E-06	7.94E-06	4.2	34.6
Adenine	136.0612	12.12	NS	NS			4.06E-03	1.90E-03	4.1	17.5
Thiamine	265.1122	10.67	NS	2.19E-02		1.6	1.13E-02	3.73E-02	2.0	2.0
Methionine	150.0592	16.17	NS	NS			4.43E-05	7.76E-05	2.2	6.4
S-adenosylhomocysteine	385.1292	13.73	NS	3.87E-02		1.5	2.68E-03	7.44E-05	1.8	NC
Trans-4-Hydroxyproline	132.0652	17.99	NS	NS			1.09E-05	1.25E-05	1.3	1.4
Phenylalanine	166.0872	16.65	NS	NS			6.82E-03	8.71E-03	1.4	2.0
Carnitine	162.1122	13.30	NS	2.15E-03		1.2	9.26E-08	1.93E-08	1.5	3.7

541

542 **Metabolites identified in CE-MS analysis of *Leishmania donovani* axenic amastigotes as being significantly**

543 **affected by miltefosine treatment in different doses/time-points. All identifications have been determined**

544 **at MSI level 1, as defined by the analysis of authentic standards. Calculated *p*-values (Student's two-tailed *t*-**

545 **test (*n=6* per group) and fold changes are shown for the lower dose (LD: 4.47 µM) or higher dose (HD: 13.41**

546 **µM) versus the un-treated samples at the respective time-point. Where *p*-values were not significant (NS: *p***

547   > 0.05), there were no fold changes to report. Fold changes are absolute; all are decreases except those

548   highlighted in grey which are calculated increases with miltefosine with respect to un-treated controls.

549   **Figures**
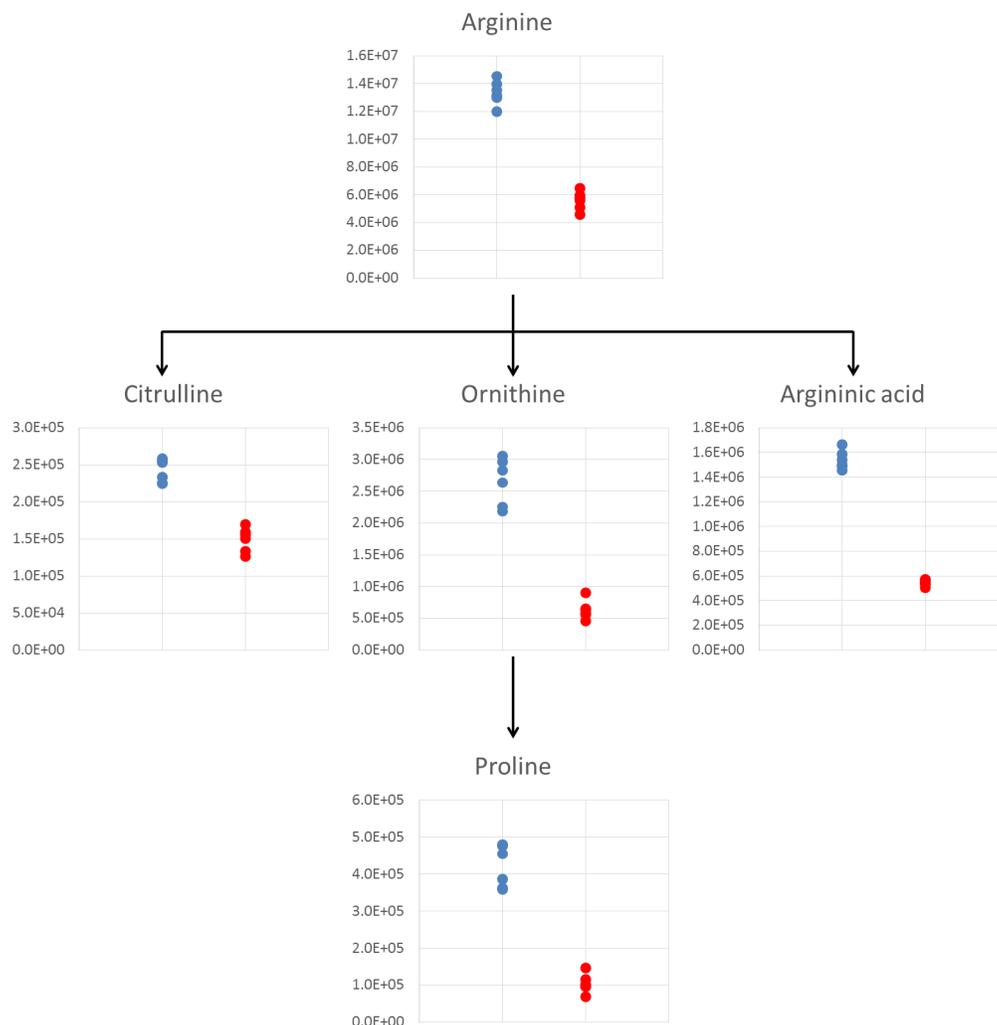
550

551   **Figure 1: Effect of miltefosine on arginine metabolism observed in *L. donovani* axenic amastigotes after 24h**

552   **of exposure at the lower dose of 4.47 μM. Plots show peak area abundances detected in samples: un-**

553   **treated parasites in blue, parasites treated with 4.47 μM miltefosine in red.**
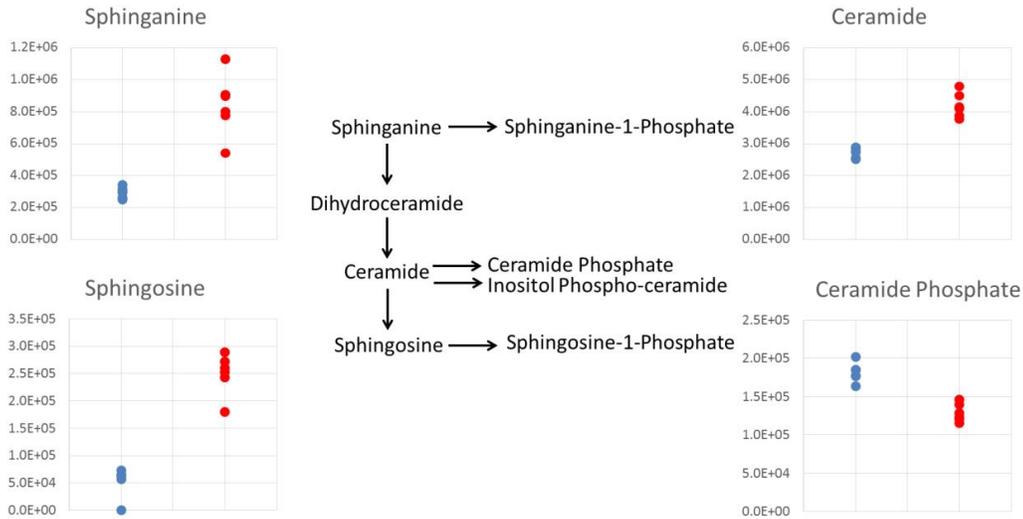
554
555 **Figure 2: Effect of miltefosine on the SL biosynthetic pathway observed in *L. donovani* axenic amastigotes**

556 **after 24h of exposure at the lower dose of 4.47 µM. Plots show peak area abundances for key SLs detected**

557 **in samples: un-treated parasites in blue, parasites treated with 4.47 µM miltefosine in red. Sphinganine**

558 **shown is C16 form, Ceramide shown is 34:1 form, Sphingosine shown is 18:1 form and ceramide phosphate**

559 **shown is 26:1 form. Plots show trends representative of all detected SLs of their type. For full list of detected**

560 **SLs, refer to Supplementary Table 3.**
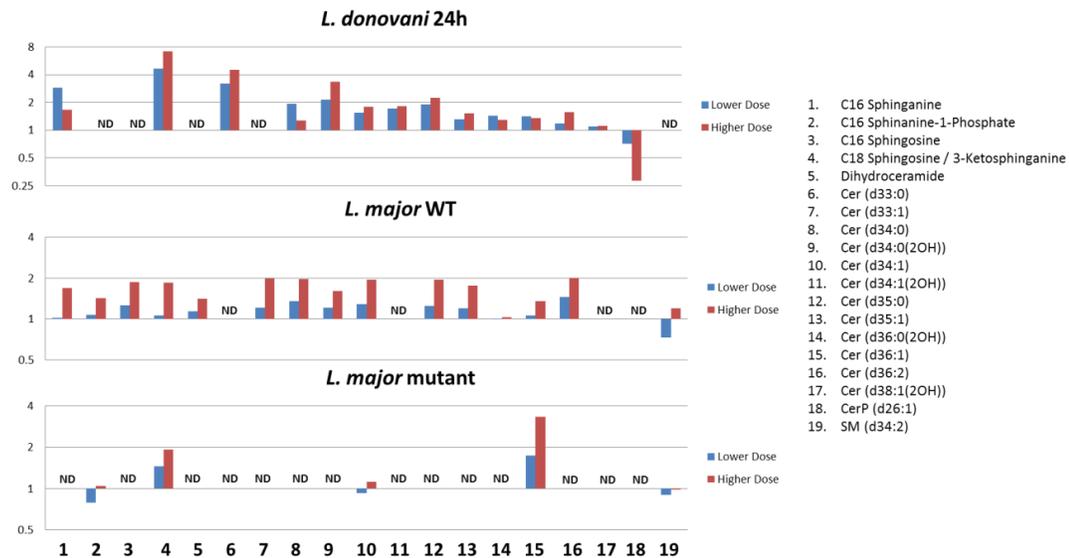
561
562 **Figure 3: Fold changes in abundance of all SLs detected in *L.donovani* axenic amastigotes and / or *L.major***

563 **promastigotes, comparing treated parasites to un-treated parasites. Lower dose and higher dose data are**

26

564 **shown for each, corresponding to 4.47 µM and 13.41 µM respectively for *L.donovani* and 10 µM and 30 µM**

565 **respectively for *L.major.* SLs that were not detected in a certain dataset are marked with 'ND', while X**

566 **denotes complete absence in drug treated parasites and presence in un-treated parasites.**
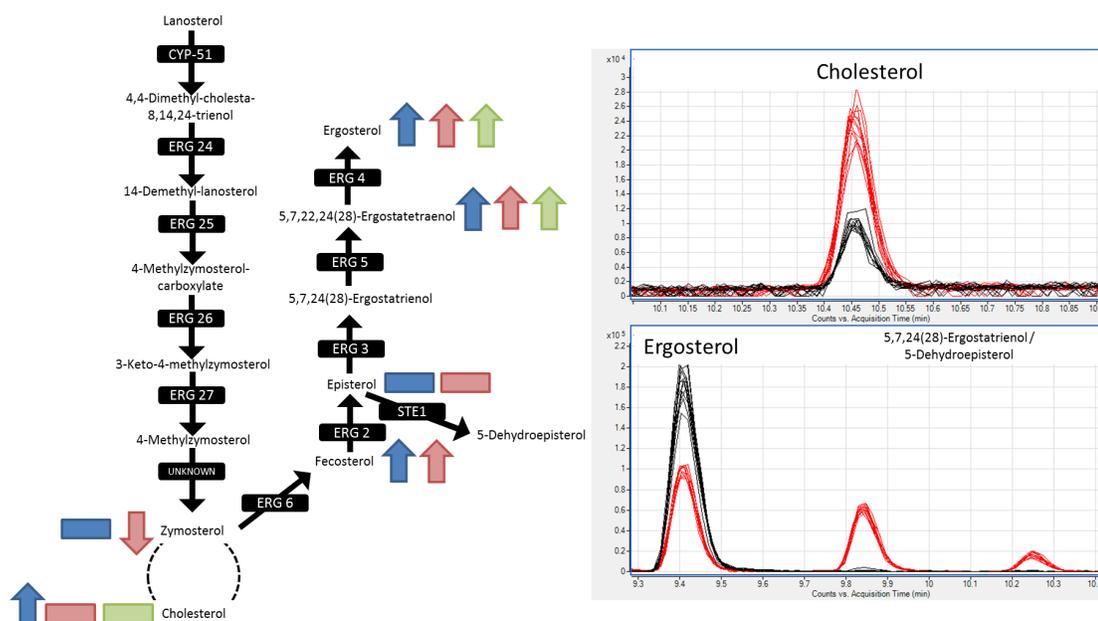
567
568 **Figure 4: Ergosterol biosynthesis pathway. Sterols increased, decreased or detected but with no change as a**

569 **response to miltefosine exposure are shown on the pathway (*L .donovani* axenic amastigotes with 5h drug**

570 **exposure in blue; *L. donovani* axenic amastigotes with 24h drug exposure in red; *L. major* wild type**

571 **promastigotes in green). In all cases, trends were seen for both low and high concentrations of miltefosine**

572 **treatment. Chromatographic peaks shown for ergosterol, cholesterol, 5,7,24(28)-Ergostatrienol and 5-**

573 **dehydroepisterol in *L. major* wildtype (black trace) and ΔLCB2 mutants (red trace) to highlight the**

574 **differences in sterol profiles between them. 5,7,24(28)-Ergostatrienol and 5-dehydroepisterol share the**

575 **same logP and therefore it is not possible to distinguish which peak (9.8-9.9 min or 10.2-10.3 min) these**

576 **sterols correspond to.**

Sphinganine

Sphingosine

Ceramide

Ceramide Phosphate

Sphinganine → Sphinganine-1-Phosphate

↓

Dihydroceramide

↓

Ceramide → Ceramide Phosphate
→ Inositol Phospho-ceramide

↓

Sphingosine → Sphingosine-1-Phosphate

L. donovani 24h

L. major WT

L. major mutant

- Lower Dose
- Higher Dose

1. C16 Sphinganine
2. C16 Sphinanine-1-Phosphate
3. C16 Sphingosine
4. C18 Sphingosine / 3-Ketosphinganine
5. Dihydroceramide
6. Cer (d33:0)
7. Cer (d33:1)
8. Cer (d34:0)
9. Cer (d34:0(2OH))
10. Cer (d34:1)
11. Cer (d34:1(2OH))
12. Cer (d35:0)
13. Cer (d35:1)
14. Cer (d36:0(2OH))
15. Cer (d36:1)
16. Cer (d36:2)
17. Cer (d38:1(2OH))
18. CerP (d26:1)
19. SM (d34:2)