Impaired development of a miltefosine-resistant *Leishmania infantum* strain in the sand fly vectors *Phlebotomus perniciosus* and *Lutzomyia longipalpis*

Lieselotte Van Bockstal\(^a\), Jovana Sádlová\(^b\), Hamide Aslan Suau\(^a\), Sarah Hendrickx\(^a\), Claudio Meneses\(^c\), Shaden Kamhawi\(^c\), Petr Volf\(^b\), Louis Maes\(^a\), Guy Caljon\(^a,\,*\)

\(^a\) Laboratory of Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Universiteitsplein 1, B-2610, Wilrijk, Belgium
\(^b\) Department of Parasitology, Charles University, Vinicna 7, CZ-12844, Prague 2, Czech Republic
\(^c\) Vector Molecular Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, United States

**Abstract**

**Objectives:** To gain insight into the propagation of miltefosine (MIL) resistance in visceral leishmaniasis, this laboratory study explored development of resistant parasites with a defective miltefosine transporter (MT) in sand flies.

**Methods:** Infectivity, colonization of stomodeal valve and metacyclogenesis of a MIL-resistant (MIL-R) *Leishmania infantum* LEM3323 line with a defective MT were assessed in the natural sand fly vectors *Phlebotomus perniciosus* and *Lutzomyia longipalpis*. Given our recent description of partial drug dependency of the MT-deficient line, the impact of MIL pre-exposure on sand fly infectivity was explored as well.

**Results:** A significant reduction in sand fly infectivity, stomodeal valve colonization and differentiation into metacyclics (determined by a lower flagellum/cell body length ratio) was observed in both vectors for MIL-R as compared to the isogenic parent MIL-susceptible line. Re-introduction of the wildtype MT gene into MIL-R tended to partially rescue the capacity to infect sand flies. Pre-exposure to MIL did not alter infectivity of the MIL-R line.

**Conclusion:** The MIL resistant *L. infantum* LEM3323 line is significantly hampered in its development and transmissibility potential in two sand fly vector species. Additional studies are warranted to evaluate whether this applies to other visceral *Leishmania* parasites with acquired MIL-resistance.

1. Introduction

Visceral leishmaniasis (VL) is a lethal neglected tropical disease caused by *Leishmania donovani* and *L. infantum* and is transmitted by the bites of infected female phlebotomine sand flies (Kamhawi, 2006). Infections by *L. donovani* occur in the Indian subcontinent, South Asia and East Africa, while *L. infantum* affects mainly populations in the Mediterranean basin and Latin America (Ready, 2014). *Lutzomyia longipalpis* and *Phlebotomus perniciosus*, the sand fly species reported in this paper, are natural vectors of *L. infantum* respectively in Latin America and the Mediterranean basin (Dvorak et al., 2018). For all *Leishmania* species developing within the sand fly, the parasites migrate anteriorly into the thoracic midgut where they differentiate into highly motile infective metacyclic promastigotes through a process called metacyclogenesis (Rogers and Bates, 2007) and which is essential for transmissibility to the mammalian host (Aslan et al., 2013; Serafim et al., 2018). The stomodeal valve is a small structure that regulates blood intake during feeding located between foregut and midgut, called the cardia. In infected sand flies, the stomodeal valve is colonized by various stages of promastigotes (Rogers et al., 2008) and transmission occurs when infective parasites are regurgitated from the cardia into the host tissue (Voil et al., 2004).

Miltefosine (MIL) (hexadecylphosphocholine) is the only oral antileishmanial drug currently available, however, its effectiveness in India and Nepal is decreasing with relapses in up to 20% of the cases (Rijal et al., 2013; Sundar et al., 2012). Because of its long half-life of about 150 h and long treatment course of 28 days, it is prone to the development of resistance (Pandey et al., 2016; Perez-Victoria et al., 2006b). Rather surprisingly, very few fully MIL-resistant (MIL-R) field isolates have yet been reported (Gojien et al., 2012; Hendrickx et al., 2016).

*Corresponding author. University of Antwerp, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences Laboratory for Microbiology, Parasitology and Hygiene (LMPH), Universiteitsplein 1, B-2610, Antwerp, Wilrijk, Belgium.

E-mail address: guy.caljon@uantwerpen.be (G. Caljon).

https://doi.org/10.1016/j.ijpddr.2019.09.003

Received 9 February 2019; Received in revised form 6 September 2019; Accepted 10 September 2019

Available online 10 September 2019

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A common feature in these MIL-R strains is a defect in the inward MIL transporter (MT) and/or its beta subunit Ro83 causing a decreased MIL accumulation (Mondelaers et al., 2016). We also recently observed that a mutation in the MT causes a severe fitness loss resulting in a compromised ability to multiply in mice. Quite unexpectedly, when this MT-defective line was exposed to MIL, parasite virulence reappeared, revealing a drug-dependent phenotype (Eberhardt et al., 2018). To obtain a better phenotypic insight into the impact of resistance acquisition, the present laboratory study evaluated the fitness of an in vitro selected MIL-R parasite line inside the natural sand fly vector.

2. Materials and methods

2.1. Ethics statement

Experiments using laboratory rodents were carried out in strict accordance with all mandatory guidelines (European Union directives, including the Revised Directive, 2010/63/EU on the Protection of Animals Used for Scientific Purposes that came into force on January 01, 2013, and the Declaration of Helsinki in its latest version) and were approved by the ethical committee of the University of Antwerp, Belgium (UA-ECD, 2017–78). Maintenance of sand fly colonies and experimental infections of sand flies in Prague were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University in Prague and were performed under permit no. MSMT-10270/2015–6 of the Ministry of the Environment of the Czech Republic.

2.2. Mice and sand flies

Female Swiss mice (20–25 g) were purchased from Janvier (France). Food for laboratory rodents (Carfil, Arendonk, Belgium) and drinking water were available ad libitum. In Prague, BALB/c mice (AnLab) were provided with a standard feed mixture ST-1 (Velaz) and water ad libitum, with a 12 h light/12 h dark photoperiod, temperature 22–25 °C and humidity 40–60%. Flies were maintained at the insectary of Charles University, Prague, Czech Republic (P. perniciosus) and at the Laboratory for Microbiology, Parasitology and Hygiene, University of Antwerp, Belgium (L. longipalpis), as described elsewhere (Volf and Volfova, 2011). In brief, flies were kept in cages at 25–26 °C and 70–80% relative humidity. Cotton wool soaked in 30% sugar solution was provided for feeding the adults. Larvae were fed three times a week with a mixture of fermented rabbit feces, rabbit pellets and mice pellets. Blood feeding of adult females was performed once weekly on anesthetized (1.75 mg/20 g ketamine and 0.25 mg/20 g xylazine) mice.

2.3. Leishmania strains

Sand fly infections were performed with (i) the parental wildtype (WT) L. infantum strain MHOM/FR/96/LEM3323, a MIL-susceptible clinical strain isolate from a French HIV co-infected patient (kindly provided by Dr. L. Lachaud, Centre National de Référence des Leishmania, Montpellier, France); (ii) the in vitro selected MIL-R LEM3323 line (Eberhardt et al., 2018; Hendrickx et al., 2015) and (iii) the episomally transfected MIL-R^LMT LEM3323 line showing restored MIL susceptibility (Mondelaers et al., 2016). Promastigotes were grown in the absence of MIL in HOMEM (Gibco®, Life Technologies, Ghent, Belgium), supplemented with 200 mM L-glutamine, 16.5 mM NaHCO₃, 10% heat-inactivated FCS, 40 mg/L adenine, 3 mg/L folic acid, 2 mg/L D-biotin and 2.5 mg/L hemin at 25 °C and were sub-cultured twice weekly. The episomally rescued MIL-R^LMT transfecant was grown under hygromycin pressure. The used WT and MIL-R parasite lines were previously subjected to a WGS-analysis (Mondelaers et al., 2016). Only one gene was found to be changed from a homozygous reference sequence to a homozygous variant (indel LinJ.13.1590 or LiMT). From the karyotype point of view, MIL-R showed a decrease in copy number of four chromosomes (1, 2, 9, 12) as compared to the WT line.

2.4. Sand fly infections

Sugar was removed from the adults 12 h before infection. Flies were fed through a chicken skin membrane with defibrinated and heat-inactivated rabbit blood (P. perniciosus) or heparinized mouse blood (L. longipalpis) containing 1 × 10⁶/mL (P. perniciosus) or 5 × 10⁶/mL (L. longipalpis) promastigotes from log-phase cultures. To study the impact of MIL pre-exposure on sand fly infectivity, MIL-R promastigotes were cultured for 1 week in HOMEM in the presence of 40 μM MIL (Carbosynth, Berkshire, UK) prior to spiking into the infectious blood meal. Only blood-fed females were used for analysis and were separated 24 h after feeding.

2.5. Sand fly dissections

Dissections were performed at days 2, 5, 9, 12 and 15 post blood meal (PBM). For each time point, parasite load and percentage infected flies were determined. The gut was dissected under a dissection microscope and collected in 50 μL DPBS (Gibco®, ThermoFisher Scientific). Subsequently, the guts were crushed with a pestle for parasite counting in a KOVA chamber. The location (abdominal midgut, thoracic midgut, cardia, stomodeal valve) and intensity of infection was inspected microscopically and scored as light (< 100 parasites), moderate (100–500 parasites) or heavy (> 500 parasites). Parasites isolated from L. longipalpis on day 9 post-infection (DPI) were cultivated in HOMEM promastigote medium supplemented with 5% penicillin-streptomycin. When sufficiently dense log-phase post-fly cultures were obtained, a standard MIL susceptibility assay was performed, as described in 2.7. All experiments were performed in at least two independent replicates.

2.6. Morphometry of parasites

At different time points PBM, the digestive tract of sand flies was dissected and the location of infection was examined microscopically. Morphometric analysis was performed on midgut smears upon fixing with methanol and staining with Giemsa. Cell body length and flagellar length of randomly selected parasites were measured using Image-J software (Schneider et al., 2012). The promastigote flagellum/cell body length ratio was determined as a measure for metacyclogenesis. Parasites with a ratio > 2 were considered as metacyclics (Zakai et al., 1998).

2.7. Drug susceptibility determination

Standard promastigote and intracellular amastigote susceptibility assays were performed to assess MIL susceptibility (Vermeersch et al., 2009). In brief, 50% inhibitory concentrations (IC₅₀) were determined by exposing log-phase promastigotes to two-fold serial dilutions of MIL starting from 40 μM. After 72 h incubation, promastigote susceptibility was determined by viability testing upon addition of resazurin and fluorescence reading (Tecan®, GENios). For amastigote susceptibility determination, primary peritoneal macrophages were harvested from starved-stimulated female Swiss mice. About 24 h after seeding, the cells were infected with stationary-phase promastigotes at a 10:1 multiplicity of infection. Residual extracellular promastigotes were removed 24 h later by washing and two-fold MIL dilutions were added. After 96 h of drug exposure at 37 °C and 5% CO₂, the plates were stained with Giemsa and IC₅₀,₆ were determined microscopically by comparing the intracellular amastigote burdens between treated and untreated cells.
A. *P. perniciosus*

B. *L. longipalpis*

C. *P. perniciosus*

D. *L. longipalpis*

E. *P. perniciosus*

F. *L. longipalpis*

G. *P. perniciosus*

H. *L. longipalpis*

(caption on next page)
2.8. Statistics

Differences in percentage infected flies and colonization of the stomodeal valve were analyzed with Chi-square ($\chi^2$) tests, parasite counts and flagellum/cell body length ratios were tested by an independent samples t-test or a one way ANOVA test in SPSS version 24. Data over multiple time points (7–14 DPBM) were also subjected to a repeated measures ANOVA with a Bonferroni correction for multiple comparisons test. Graphs were made with GraphPad Prism.

3. Results

3.1. MIL-R parasites are hampered in their development in P. perniciosus and L. longipalpis

The MIL-R line, harboring the MT-deficiency and a decreased copy number of four chromosomes, was previously shown to display a decreased rate of in vitro promastigote growth and metacyclogenesis (Eberhardt et al., 2018; Hendrickx et al., 2016). In sand flies, the MIL-R line was significantly impacted in its infectivity as compared to the isogenic WT line (Fig. 1). At 9 days PBM, a significant difference in percentage of infected flies was observed, which was higher for L. longipalpis (WT 79.9% - MIL-R 24.7%) as compared to P. perniciosus (WT 33.3% - MIL-R 10.2%) (Fig. 1A and B). The proportion of heavy and moderate infections tended to be higher amongst infected flies at 5 days PBM with WT parasites [L. longipalpis (WT 88.9% - MIL-R 41.7%, p = 0.014) and P. perniciosus (WT 100.0% - MIL-R 70.0%, p = 0.052)] but was not significantly different at 9 dpi [L. longipalpis (WT 78.6% - MIL-R 66.7%, p = 0.251) and P. perniciosus (WT 85.7% - MIL-R 80.0%, p = 0.624)] (Fig. 1C and D). When measuring the flagellar/cell body length ratio as indicator for metacyclogenesis, the MIL-R line displayed a significantly lower flagellum/cell body length ratio at 9 days PBM compared to the WT in L. longipalpis (WT 1.31 ± 0.03 μm - MIL-R 1.17 ± 0.03 μm) and in P. perniciosus (WT 1.37 ± 0.03 μm - MIL-R 1.20 ± 0.02 μm) (Fig. 1E and F). The percentage of metacyclic parasites at 9 DPBM per infected fly were calculated to be lower for the MIL-R strain (L. longipalpis 4.9% and P. perniciosus 5.5%) compared to the WT strain (L. longipalpis 10.7% and P. perniciosus 8.3%). Since colonization of the stomodeal valve is prerequisite for transmission potential to the mammalian host (Rogers and Bates, 2007), the location of infection inside both vectors was investigated (Fig. 1G and H): WT more efficiently (p < 0.05) colonized the stomodeal valve (P. perniciosus 14.3% - L. longipalpis 46.7%) compared to MIL-R (P. perniciosus 2.0% - L. longipalpis 4.9%). Repeated measure ANOVA with Bonferroni multiple comparisons test of the data over multiple time points (7–15 DPBM) of all experiments performed in L. longipalpis (Fig. 2), revealed a significantly hampered infection of the MIL-R line for all infection parameters assessed: percentage infected flies (p < 0.001), parasite load (p < 0.0001), stomodeal valve colonization (p < 0.05) and metacyclogenesis (p < 0.01).

3.2. Episomal reconstitution of MIL-R with WT LiMT restores the metacyclogenesis inside both vectors

Introducing the WT transporter into the MIL-R line tended to cause a limited recovery of infectivity in L. longipalpis (Fig. 2). At the different time points PBM, a trend for an intermediate phenotype in terms of percentage infected flies was observed for MIL-R$^{\text{LiMT}}$ (Fig. 2A). The actual numbers of parasites were not significantly higher compared to the MIL-R mutant (Fig. 2B). Focusing on colonization of the stomodeal valve, the tendency of an increase was observed for MIL-R$^{\text{LiMT}}$ at 9–15 days PBM in L. longipalpis (Fig. 2C), suggesting that once the infection becomes established, the MT-protein promotes parasite survival enabling colonization of the stomodeal valve. Although no significant differences were observed for the parameters discussed above, introduction of a wildtype LiMT gene seemed to increase metacyclogenesis considering all experiments performed (L. longipalpis 9.4% - P. perniciosus 12.1% at 9 DPBM) with significantly increased flagellum/cell body length ratios (L. longipalpis 1.29 ± 0.04 μm, p < 0.05, Fig. 2D - P. perniciosus 1.40 ± 0.03 μm, p < 0.0001, Fig. S1C), suggesting that MT plays a role during infection in L. longipalpis and P. perniciosus.

To evaluate the stability of the MIL susceptibility profile of the WT, MIL-R and MIL-R$^{\text{LiMT}}$ lines following passage in the sand fly (without hygromycin selection of the episomal LiMT copy), susceptibility was determined on post-fly isolates at the promastigote and intracellular amastigote stage. Passage through the vector did not affect MIL susceptibility of the LiMT-deficient MIL-R line. Moreover, the episomally rescued MIL-R$^{\text{LiMT}}$ line remained susceptible in absence of hygromycin pressure (Table 1).

3.3. Miltefosine pre-treatment of the MIL-R line does not significantly alter sand fly infectivity

Given the previously described drug-dependency of the MIL-R line (Eberhardt et al., 2018), the effect of in vitro pretreatment of parasites with 40 μM MIL on infection and development inside L. longipalpis was evaluated. No significant differences in parasite load, metacyclic load and colonization of the stomodeal valve were observed between the two groups (Fig. 3).

4. Discussion

This laboratory study focused on MIL resistance of visceral Leishmania parasites to characterize phenotypic effects in the sand fly vector that may affect some essential aspects of parasite transmissibility. Miltefosine is currently the only oral drug for visceral leishmaniasis and although deficiency in the aminophospholipid/MIL transporter is sufficient to elicit full resistance, only very few naturally MIL-resistant parasites appear to circulate. The two isogenic L. infantum LEM3323 lines with a differential MIL susceptibility used in this study differed by a lower copy number of four chromosomes (1, 2, 9, 12) and by the presence of a 2 bp-deletion in the LiMT gene, leading to an early stop codon and inactivation of the LiMT protein (Mondelaers et al., 2016). LiMT is involved together with its β-subunit R03 in phospholipid translocation across the plasma membrane (Perez-Victoria et al., 2006a). A low expression or a defect of this transporter will impact MIL uptake and convey resistance (Garcia-Sanchez et al., 2014). The parasitological effects of the defective drug transporter were previously characterized in vitro and in experimental rodent infections (Eberhardt et al., 2018; Hendrickx et al., 2016).

The phenotypic impact of MIL resistance in the selected parasite line was studied in L. longipalpis and P. perniciosus, both natural vectors of L.
infantum (Dvorak et al., 2018; Sacks and Kamhawi, 2001). Despite higher infection rates in L. longipalpis compared to P. perniciosus, development of the isogenic L. infantum lines showed the same relative differences. Although infection levels in both species of sand flies are relatively low, they are sufficient to appreciate the overall hampered development of the MIL-R line. Indeed, a significant fitness loss of MIL-R was recorded as compared to the WT counterpart, at the level of sand fly infectivity, stomodeal valve colonization and rate of metacyclogenesis. In both vectors, there was a decrease in parasite load from day 2 to day 5 post-infection due to the early events of blood meal digestion during which the parasites need to overcome expulsion during defecation. Additionally, parasite loads on days 7–12 post-infection were lower for MIL-R compared to WT, although high midgut loads may not necessarily be a prerequisite for successful transmission (Aslan et al., 2013; Secundino et al., 2012). Nevertheless, colonization of the stomodeal valve and metacyclogenesis, both critical for transmission, were significantly impacted in L. longipalpis and P. perniciosus. This indicates that the studied MIL-R line may be poorly transmissible. It would be interesting to investigate the development of other MIL-R Leishmania strains inside the insect vector. Beside mutations in the MT gene, other mechanisms may cause an elevated MIL resistance, such as mutations in the ROS3 gene encoding a subunit of the MIL transporter complex or overexpression of ABC transporters resulting in increased drug efflux (Dorlo et al., 2012). It remains to be evaluated how such changes would influence the parasite’s ability to infect and develop within the sand fly vector.

Table 1
Promastigote and amastigote susceptibility of the selected strains against MIL before and after passage in L. longipalpis. Results are based on two independent repeats run in triplicate and are expressed as mean IC50 value (μM) ± standard error of the mean (SEM).

| Strains     | Promastigote IC50 ± SEM (μM) | Post-fly promastigote IC50 ± SEM (μM) | Intracellular amastigote IC50 ± SEM (μM) | Post-fly amastigote IC50 ± SEM (μM) |
|-------------|-----------------------------|--------------------------------------|-----------------------------------------|------------------------------------|
| LEM3323 WT  | 0.93 ± 0.86                 | 1.71 ± 0.23                          | 0.52 ± 0.04                             | 0.63 ± 0.11                        |
| LEM3323 MIL-R | > 40.00                    | > 40.00                               | > 20.00                                 | > 20.00                            |
| LEM3323 MIL-R LiMT | 0.19 ± 0.08               | 0.35 ± 0.15                          | 0.62 ± 0.20                             | 0.48 ± 0.06                        |

Fig. 2. Infection and development of the MIL-R and episomally reconstituted MIL-RLiMT parasites in L. longipalpis (A-D). Infection parameters for WT, MIL-R and MIL-RLiMT L. infantum lines. (A) Infection rates (% infected females) at different days post blood meal (DPBM). Number of dissected females are shown above bars. (B) Parasite load in the gut of infected female sand flies over time. Number of dissected females are shown above bars. Results are expressed as mean ± SEM. (C) Percentage of females with a colonized stomodeal valve over different time points post blood meal. Number of dissected females are shown above bars. (D) Metacyclogenesis of parasites in the gut of infected females. Results are expressed as mean ± SEM. Number of parasites that were measured are shown above bars. Significance levels based on a repeated measure ANOVA with Bonferroni multiple comparisons test are indicated next to the legends. Significance levels within experiments are indicated with a black asterisk for WT-MIL-R comparisons and with a red asterisk for MIL-R-MIL-RLiMT comparisons (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Fig. 3. Effect of in vitro MIL pretreatment on infectivity and development of MIL-R in L. longipalpis. (A) Infection rate at different days post blood meal (DPBM) of MIL-R and MIL-R pretreated at 40 μM MIL. Number of dissected females are shown above bars. (B) Parasite load during the development of MIL-R in L. longipalpis. Number of dissected females are shown above bars. (C) Percentage of female sand flies with a colonized stomodeal valve at different DPBM. Number of dissected females are shown above bars. (D) Metacyclogenesis of parasites in the gut of infected females at day 9 DPBM. Results are expressed as mean ± SEM. Number of parasites that were measured are shown above bars. Results are based on two independent repeats. Differences between groups were evaluated using an independent samples t-test or Chi-square test.

infantum (Dvorak et al., 2018; Sacks and Kamhawi, 2001). Despite higher infection rates in L. longipalpis compared to P. perniciosus, development of the isogenic L. infantum lines showed the same relative differences. Although infection levels in both species of sand flies are relatively low, they are sufficient to appreciate the overall hampered development of the MIL-R line. Indeed, a significant fitness loss of MIL-R was recorded as compared to the WT counterpart, at the level of sand fly infectivity, stomodeal valve colonization and rate of metacyclogenesis. In both vectors, there was a decrease in parasite load from day 2 to day 5 post-infection due to the early events of blood meal digestion during which the parasites need to overcome expulsion during defecation. Additionally, parasite loads on days 7–12 post-infection were lower for MIL-R compared to WT, although high midgut loads may not necessarily be a prerequisite for successful transmission (Aslan et al., 2013; Secundino et al., 2012). Nevertheless, colonization of the stomodeal valve and metacyclogenesis, both critical for transmission, were significantly impacted in L. longipalpis and P. perniciosus. This indicates that the studied MIL-R line may be poorly transmissible. It would be interesting to investigate the development of other MIL-R Leishmania strains inside the insect vector. Beside mutations in the MT gene, other mechanisms may cause an elevated MIL resistance, such as mutations in the ROS3 gene encoding a subunit of the MIL transporter complex or overexpression of ABC transporters resulting in increased drug efflux (Dorlo et al., 2012). It remains to be evaluated how such changes would
be tolerated in the sand fly vector.

The observed fitness loss of the studied MIL-R line could be partially restored, with a significant impact on metacyclogenesis, by introducing an episcopal copy of the wildtype $\text{MT}^\text{g}$ gene. These observations suggest that the MT transporter is important for the maintenance of the plasma membrane composition (Fernandez-Prada et al., 2016; Perez-Victoria et al., 2006b; Rakotomanga et al., 2005) and thereby impacts the infection process in the sand fly vector. These results mirror observations made previously in mice, where the MIL-R line displayed a severely compromised potential to colonize visceral organs and where episomal MT reconstitution is able to partially restore parasite virulence (Eberhardt et al., 2018). A possible explanation why an intermediate phenotype is observed after re-introduction of the WT MT gene, is the difference in regulation of gene expression between the episomal and chromosomal genes and the absence of hygromycin selection pressure during the in vivo studies. Alternatively, the impact of the somy differences between the MIL-R and WT isogenic lines (www.addn.be) and the Excellence Centre LMPH is a partner of the Antwerp Drug Discovery Network (ADDN, 2018). Analogous experiments in sand flies where the infectious blood meal was supplemented with the drug, did not show a significant impact on parasite development. Collectively, this study shows that a MIL resistant parasite line is significantly hampered in its infectivity for two sand fly species. Additional studies on a larger panel of MIL-R parasite strains is essential to understand the transmissibility potential in view of the limited number of fully MIL-resistant clinical VL isolates identified so far.

Acknowledgements

This work was funded by the Fonds Wetenschappelijk Onderzoek Vlaanderen(FWO No. 1136417N (L.V.B.), 1201037N (S.H.)), Research Funds of the University of Antwerp (TT-ZAPBOF 33049 (G.C.) and TOP-BOF 35017) and by COST-STM (CM1307-050317-081118). J.S. and P.V. were supported by an ERD Funds, project CePvIP VI.019/0000759. The authors thank Laurence Lachaud (Laboratoire de Parasitologie-Mycologie and Centre National de Référence des Leishmanioses, Montpellier, France) for providing the LEM3323 clinical isolate. The technical assistance of Mandy Vermont and Maarten Van Den Bogaerde during the sand fly experiments is highly appreciated. LMPH is a partner of the Antwerp Drug Discovery Network (ADDN, www.addn.be) and the Excellence Centre ‘Infa-Med’ (www.uantwerpen.be/infra-med).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ipddr.2019.09.003.

References

Aslan, H., Dey, R., Meneses, C., Castrovinci, P., Jeronimo, S.M., Oliva, G., Fischer, L., Duncan, R.C., Nakhati, H.L., Valenzuela, J.G., Kambahi, S., 2013. A new model of progressive visceral leishmaniasis in hamsters by natural transmission via bites of vector sand flies. J. Infect. Dis. 207, 1328–1338.

Cojean, S., Houze, S., Hauchecine, D., Huteau, F., Lariven, S., Hubert, V., Michard, P., Bories, C., Pratfong, L., Le Bras, J., Loeuille, P.M., Mathevon, S., 2012. Leishmania resistance to miltefosine associated with genetic marker. Emerg. Infect. Dis. 18, 704–706.

Dorlo, T.P., Balasegaram, M., Beijnen, J.H., de Vries, P.J., 2012. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. J. Antimicrob. Chemother. 67, 2576–2597.

Dvorak, V., Shaw, J., Volf, P., 2018. Parasite biology: the vectors. In: Bruschi, F., Gradoni, L. (Eds.), The Leishmanias: Old Neglected Tropical Diseases. Springer, pp. 31–77.

Eberhardt, E., Bulle, D., Van Boekstal, L., Van den Kerkhof, M., Cos, P., Delputte, P., Hendrickx, S., Maes, L., Caljon, G., 2019 Feb 1. Miltefosine enhances the fitness of a non-virulent drug-resistant Leishmania infantum strain. J Antimicrob. Chemother. 74 (2), 395–406. https://doi.org/10.1093/jac/dky450.

Fernandez-Prada, C., Vincent, I.M., Brotherton, M.C., Roberts, M., Roy, G., Rivas, L., Leprohon, P., Smith, T.K., Ouellette, M., 2016. Different mutations in a $\gamma$-type ATPase transporter in Leishmania parasites are associated with cross-resistance to two leading drugs by distinct mechanisms. PLoS Neglected Trop. Dis. 10, e0005171.

Garcia-Sanchez, S., Sanchez-Canete, M.P., Gamarro, F., Castany, S., 2014. Functional role of evolutionarily highly conserved residues. N-glycosylation level and domains of the Leishmania miltefosine transporter-GeD50 subunit. Biochem. J. 459, 83–94.

Hendrickx, S., Beijers, J., Mondelers, A., Eberhardt, E., Lachaud, L., Delputte, P., Cos, P., Maes, L., 2016. Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in Leishmania infantum. J. Antimicrob. Chemother. 71, 1914–1921.

Hendrickx, S., Boulet, G., Mondelers, A., Dujardin, J.C., Rijal, S., Lachaud, L., Cos, P., Delputte, P., Maes, L., 2014. Experimental selection of promycomin and miltefosine resistance in intracellular amastigotes of Leishmania donovani and L. infantum. Parasitol. Res. 113, 1875–1881.

Hendrickx, S., Mondelers, A., Eberhardt, E., Lachaud, L., Delputte, P., Cos, P., Maes, L., 2015. Intracellular amastigote replication may not be required for successful in vitro selection of miltefosine resistance in Leishmania infantum. Parasitol. Res. 114, 2561–2565.

Kamhawi, S., 2006. Phlebotomine sand flies and Leishmania parasites: friends or foes? Trends Parasitol. 22, 439–445.

Mondelers, A., Sanchez-Canete, M.P., Hendrickx, S., Eberhardt, E., Garcia-Hernandez, R., Lachaud, L., Cotton, J., Sanders, M., Cuypers, B., Imamura, H., Dujardin, J.C., Delputte, P., Cos, P., Caljen, G., Gamarro, F., Castany, S., Maes, L., 2016. Genomic and molecular characterization of miltefosine resistance in Leishmania infantum strains with either natural or acquired resistance through experimental selection of intramacostigotes. PLoS One 11, e0154101.

Pandey, K., Ravidas, V., Siddiqui, N.A., Sinha, S.K., Verma, R.B., Singh, T.P., Dharwahl, A.C., Das Gupta, R.K., Das, P., 2016. Pharmacovigilance of miltefosine in treatment of visceral leishmaniasis in endemic areas of Bihar, India. Am. J. Trop. Med. Hyg, 95, 1100–1105.

Perez-Victoria, F.J., Sanchez-Canete, M.P., Castany, S., Gamarro, F., 2006a. Phospholipid translocation and miltefosine potency require both L. donovani miltefosine transporter and the new protein Ld100 in Leishmania parasites. J. Biol. Chem. 281, 23766–23775.

Perez-Victoria, F.J., Sanchez-Canete, M.P., Seifert, K., Croft, S.L., Sundar, S., Castany, S., Gamarro, F., 2006b. Mechanisms of experimental resistance of Leishmania to miltefosine: implications for clinical use. Drug Resist. Updates 9, 26–39.

Rakotomanga, M., Saint-Pierre-Chaazlet, M., Loiseau, P.M., 2005. Alteration of fatty acid and sterol metabolism in miltefosine-resistant Leishmania donovani promastigotes and consequences for drug-membrane interactions. Antimicrob. Agents Chemother. 49, 2677–2686.

Rexdy, P.D., 2014. Epidemiology of visceral leishmaniasis. Clin. Epidemiol. 6, 147–154.

Rijal, S., Ostyn, B., Uraw, S., Rai, K., Bhattari, N.R., Dorlo, T.P., Beijnen, J.H., Vanaerschot, M., Decuyper, S., Dhakal, S.S., Das, M.L., Karpi, P., Singh, R., Boelaert, M., Dujardin, J.C., 2013. Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or non-compliance. Clin. Infect. Dis. 55, 1530–1538.

Rogers, M.E., Bates, P.A., 2007. Leishmania manipulation of sand fly feeding behavior results in enhanced transmission. PLoS Pathog. 3, e91.

Rogers, M.E., Hajmova, M., Joshi, M.B., Sadlova, J., Dwyer, D.M., Volf, P., Bates, P.A., 2008. Leishmania chinatae facilitates colonization of sand fly vectors and enhances transmission to mice. Cell Microbiol. 10, 1363–1372.

Sacks, D., Kamhawi, S., 2001. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. Annu. Rev. Microbiol. 55, 453–483.

Schneider, C., Rasbash, W., Eliktier, C., 2012. NIH Image to Image: 25 years of Image Analysis. Nat Methods 9 (7), 671–675. https://doi.org/10.1038/nmeth.2089.

Secundo, N.F., de Freitas, V.C., Monteiro, C.C., Pires, A.C., David, B.A., Pimenta, P.F., 2012. The transmission of Leishmania infantum chagasi by the bite of the Lutzomyia longipalpis to two di...