Universiteit Antwerpen

This item is the archived peer-reviewed author-version of:

Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in Leishmania infantum

Reference:

Hendrickx Sarah, Beyers J., Mondelaers Annelies, Eberhardt Eline, Lachaud L., Delputte Peter, Cos Paul, Maes Louis.- Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in Leishmania infantum

The journal of antimicrobial chemotherapy - ISSN 0305-7453 - 71:7(2016), p. 1914-1921

Full text (Publishers DOI): http://dx.doi.org/doi:10.1093/JAC/DKW096

To cite this reference: http://hdl.handle.net/10067/1328240151162165141

uantwerpen.be

Institutional repository IRUA

ORIGINAL ARTICLE

Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in *Leishmania infantum*

Hendrickx S[1], Beyers J[1], Mondelaers A[1], Eberhardt E[1], Lachaud L[2], Delputte P[1], Cos P[1], Maes L[1#]

[1] Laboratory of Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Antwerp, Belgium

[2] Laboratoire de Parasitologie-Mycologie et Centre National de Référence des Leishmanioses, Centre Hospitalier Universitaire et Université de Montpellier, France.

Running title – Impact of drug resistance on *Leishmania* parasite fitness

Keywords: *in vitro, in vivo*, promastigote, amastigote, fitness, drug-resistance

[#] Corresponding Author's Address
Prof. dr. Louis Maes
University of Antwerp
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
Laboratory of Microbiology, Parasitology and Hygiene (LMPH)
Universiteitsplein 1 – building S 7.27, B-2610 Antwerp, Wilrijk (Belgium)
Tel: (32)3/265.26.27 - Fax: (32)3/265.26.81
*E-mail: louis.maes@uantwerpen.be

Synopsis

OBJECTIVES: Although miltefosine and paromomycin were only recently introduced to treat visceral leishmaniasis, increasing numbers of miltefosine treatment failures and occasional primary resistance against both drugs have been reported. Understanding alterations in parasite behaviour linked to drug-resistance is essential to assess the propensity for emergence and spread of resistant strains, particularly since a positive effect on fitness has been reported for antimony-resistant parasites. This laboratory study compared the fitness of a drug-susceptible parent wild-type (WT) clinical *Leishmania infantum* isolate (MHOM/FR/96/LEM3323) and derived miltefosine and paromomycin drug-resistant lines that were experimentally selected at intracellular amastigote level.

METHODS: Parasite fitness of WT, paromomycin- and miltefosine-resistant strains, *in vitro* and *in vivo* parasite growth, metacyclogenesis, infectivity and macrophage stress responses were comparatively evaluated.

RESULTS: No significant differences in promastigote fitness were noted between the WT and paromomycin-resistant strain, while clear benefits could be demonstrated for paromomycin-resistant amastigotes in terms of enhanced *in vitro* and *in vivo* growth potential and intracellular stress response. The miltefosine-resistant phenotype showed incomplete promastigote metacyclogenesis, a decreased intracellular growth and weakened stress response, revealing a reduced fitness compared to WT parent parasites.

CONCLUSIONS: The rapid selection and fitness advantages of paromomycin-resistant amastigotes endorse the current use of paromomycin in combination therapy. Although a reduced fitness of miltefosine resistant strains may explain the difficulty of miltefosine resistance selection *in vitro*, the growing number of miltefosine treatment failures in the field still requires further exploratory research.

Introduction

The spread of primary antimony resistance in the Indian subcontinent has enforced the introduction of miltefosine and paromomycin for the treatment of visceral leishmaniasis (VL). Despite the increasing number of miltefosine treatment failures,[1] clinical reports on primary miltefosine or paromomycin field resistance are still very scarce.[2, 3] Contrary to *Leishmania donovani*, miltefosine relapse isolates from *L. infantum* infected patients display a decreased susceptibility, which may possibly also be related to its veterinary use to treat canine leishmaniasis and *L. infantum*-associated HIV-coinfections. Since incomplete parasite eradication is the rule in both groups, the large residual parasite reservoir will promote selection of miltefosine resistance upon repeated drug exposure.[2-4] To support strategies concerning treatment and emergence of drug resistance, experimental selection of drug resistance can facilitate applied and fundamental 'drug resistance' research with the particular advantage that the drug susceptible wild type (WT) can be directly compared with matched derived resistant lines. While in the past resistance has mostly been selected on promastigotes, an earlier study by our group demonstrated that selection of drug resistance strongly depends on the selection protocol leading to the recommendation to use intracellular amastigotes whenever possible.[5] Although former research mainly focused at unraveling resistance mechanisms, parasite fitness must be considered a relevant factor as well, potentially influencing the spreading potential of resistant strains. Comparison of unmatched antimony-susceptible and antimony-resistant *L. donovani* strains from the Indian subcontinent indicated an enhanced fitness of antimony resistant isolates.[6-11] Likewise, a large-scale field study on *L. donovani* miltefosine cure and relapse isolates from Nepal suggested a higher *in vitro* infectivity of miltefosine relapse isolates.[12] Recently, evidence for increased fitness was obtained after selection of paromomycin resistance on *L. donovani* promastigotes.[13]

The present laboratory study aimed to evaluate the impact of experimental miltefosine and paromomycin resistance on parasite fitness in a *L. infantum* strain isolated from a HIV co-infected

patient.[14] Next to promastigote growth and metacyclogenesis, the *in vitro* and *in vivo* amastigote growth pattern and the intracellular stress response upon macrophage stimulation were compared between the drug-susceptible parent WT and the miltefosine-resistant and paromomycin-resistant (R) derived strains. While a decreased fitness could be demonstrated for the miltefosine-R strain, the paromomycin-R isolate displayed enhanced intracellular amastigote growth and survival.

Materials and methods

Parasite strains: The *L. infantum* field isolate (MHOM/FR/96/LEM3323) used for the experimental selection of both miltefosine and paromomycin resistance was obtained from the 'Centre National de Référence des Leishmania (CNRL)' and was isolated from a French HIV patient.[14] Promastigotes were grown in HOMEM medium (Gibco®, Life technologies, Ghent, Belgium) at 25°C and sub-cultured twice weekly. Resistance was selected on intracellular amastigotes as previously described.[5] Promastigote and amastigote susceptibilities were determined as previously described [15] and are summarized in Table 1.

Promastigote growth: The growth profile of WT, miltefosine-R and paromomycin-R promastigotes was assessed by flow cytometry (FCM). Promastigotes were diluted in phosphate buffered saline (PBS) (Gibco®, Life technologies, Ghent, Belgium) for FCM counting, using a FACSCalibur® flow cytometer (BD Biosciences, NJ, USA) with addition of CountBright absolute counting beads (CB; Molecular Probes®, OR, USA) as internal standard for quantification of the exact volume analyzed. To generate growth curves, promastigotes of each strain were inoculated in 5 mL HOMEM at exact $5x10^5$ promastigotes/mL. Every 24h, three biological replicates were quantified *in duplo* for up to 240h and analyzed using the BD CellquestPro® software. The average promastigote density at each time point was calculated and used to draw the final growth curves (Fig. 1).

Promastigote metacyclogenesis: Promastigote morphology was evaluated microscopically to assess metacyclogenesis (Fig. 2). The promastigote flagellum/cell body length ratio was determined and promastigotes were considered metacyclic when this ratio exceeded 2.[9] Starting from 96h-old cultures and complementary to FCM assessment of promastigote density, a drop of promastigote suspension was Giemsa-stained every 24h and visualized with bright field microscopy (Axiovert 200m®, Carl Zeiss) using the Zeiss Axiocam MRm®. The flagellum/cell body length ratio of at least 50 promastigotes was determined using the Axiovision® software.

Promastigote infectivity: To evaluate the *in vitro* infectivity of WT, paromomycin-R and miltefosine-R promastigotes, their macrophage infection potential was determined.[16] Promastigotes were counted by FCM and used to infect primary mouse peritoneal macrophages at an infection ratio of 5/1. Live/dead staining with TO-PRO®-3 iodide (Molecular probes®, OR, USA) was used to correct for the variable number of dead promastigotes in the different cultures.[17] Twenty-four hours post-infection, the macrophages were fixed with methanol, Giemsa-stained and light microscopically evaluated for level of infection. To determine the number of intracellular amastigotes per macrophage and the percentage of infected macrophages, the intracellular amastigote burden of 100 macrophages was counted in 10 randomly selected microscopic fields and used to calculate the infection index:

$$\text{Infection index} = \frac{total\ \#\ amastigotes\ counted}{total\ \#\ cells\ counted} \times total\ \#\ macrophages$$

In vitro* intracellular amastigote growth:* To evaluate the intracellular amastigote growth of WT, paromomycin-R and miltefosine-R parasites, primary peritoneal mouse macrophages were collected from female Swiss mice and seeded in 96-well plates as described earlier.[18] Twenty-four hours later, promastigotes were used for infection of macrophages adopting the above metacyclogenesis criteria and following quantification by FCM. Evaluation of amastigote replication was performed every 24h by light microscopic determination of the average infection index of minimum 50 Giemsa-stained macrophages. To allow comparison between the different strains, a correction for the baseline

infectivity was made based on the infection ratio at 24h post-infection (T0). The following formula was used to calculate the amastigote multiplication ratio:

$$\text{Amastigote multiplication ratio} = \frac{\#\ amastigotes\ at\ Tx}{\#\ amastigotes\ at\ T0}$$

***In vivo* amastigote multiplication**: To evaluate *in vivo* infectivity and growth of each strain, twelve female Balb/c mice were infected intracardially with 2 x 10^7 metacyclic promastigotes. Up to 28 days post-infection, 3 animals per group were sacrificed at weekly intervals to determine the parasite burdens in liver, spleen and bone-marrow. Amastigote burdens are expressed as Leishman-Donovan Units (LDU) after microscopic quantification of the Stauber index,[19] and by SYBR Green-based Real-Time PCR targeting the cysteine protease b (*cpb*) gene. Both the forward primer (5'-ATG TCTTAC CAG AGC GGC G-3') and the reverse primer (5'-TCA CCC CAC GAG TTC TTG AT-3') were purchased from integrated DNA technologies (Leuven, Belgium). To assess amastigote viability, a small piece of organ was placed in HOMEM medium and incubated at 25°C for 2 weeks to assess promastigote back-transformation.

Intracellular amastigote stress resistance: To evaluate the capacity of intracellular amastigotes to cope with intracellular stress, infected macrophages were exposed to either *E. coli* derived LPS and IFN-γ at concentrations ranging from 0.05 to 100 ng/mL (Sigma-Aldrich, Diegem, Belgium) or with S-nitroso-N-acetyl-DL-penicillamine (SNAP) (Sigma-Aldrich, Diegem, Belgium) at concentrations ranging from 0 to 800 µM for 48h, as described earlier.[7, 13, 20, 21] Macrophage stress responses were determined by microscopic assessment of the percentage amastigote burden reduction compared to unstimulated infected control cells. Since previous research on WT and paromomycin-R strains suggested differences in host cell IL-10 production upon stimulation,[13] the production of endogenous IL-10 in the supernatant upon stimulation with 100 ng/mL LPS and 5 ng/mL IFN-γ [21] was measured using ELISA (eBioscience, Vienna, Austria).[13] To correct for the variable infection ratio between strains and the associated differences in IL-10 production, the ratio of IL-10 production of stimulated infected cells was compared to unstimulated infected cells with correction for possible background IL-10 production.

Statistical analysis: All statistical analyses were performed using Graphpad Prism version 4.00 software. Statistical differences between WT and R parasites and between the different time points within one group were determined using 2-way ANOVA with Bonferroni post-hoc comparisons for parasite growth, parasite morphology and infection indices. Morphological and infection indices intergroup comparisons were done using non-parametric Friedman test followed by Dunn's post-hoc comparisons. Tests were considered statistically significant if $p < 0.05$.

Ethical statement: The use of laboratory rodents was carried out in strict accordance to all mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes that came into force on 01/01/2013, and the declaration of Helsinki in its latest version) and was approved by the ethical committee of the University of Antwerp, Belgium (UA-ECD 2010–17 (18-8-2010).

Results

Promastigote growth: No statistical differences could be demonstrated between paromomycin-R and WT parasites (Fig. 1A), whereas the miltefosine-R strain showed a significantly decreased growth pattern starting after 96h of cultivation (Fig. 1B). All strains reached the stationary-phase leading to metacyclogenesis at around 144h.

Promastigote metacyclogenesis: Although some significant differences could be observed for WT and paromomycin-R parasites at 144h and 168h in favor of paromomycin-R, the predetermined metacyclogenesis cut-off value for both strains was reached after 192h. At that time point, no differences in percentage metacyclics (about 64%) was observed between WT and paromomycin-R parasites (Fig. 2A). The metacyclogenesis process in miltefosine-R parasites was much less evident (Fig. 2B) and did not exceed 16% (data not shown). Despite several efforts to enhance metacyclogenesis and

thus increase infectivity of the miltefosine-R strain by adaptations to the culture medium and enforced metacyclogenesis by promastigote preconditioning,[18] no increase in percentage metacyclic promastigotes could be obtained (data not shown).

Promastigote infectivity: All strains reached their highest infectivity after 144h of cultivation. At that time point, the infection indices of WT and paromomycin-R parasites were not statistically different (Fig. 3A), whereas the infection index of the miltefosine-R strain was markedly lower (Fig. 3B), corresponding to its lower level of metacyclogenesis.

***In vitro* intracellular amastigote growth**: Although an initial increase in intracellular amastigote burden was observed for all strains, paromomycin-R amastigotes did show a notable advantage over WT and miltefosine-R parasites (Fig. 4).

***In vivo* amastigote multiplication**: Female Balb/c mice were infected with WT, miltefosine-R and paromomycin-R promastigotes for comparative monitoring of the amastigote multiplication ratio in the target organs. Every 7 days, three mice per strain were sacrificed and intracellular amastigote burdens in liver and spleen were determined by microscopic counting and real-time PCR. As bone-marrow yields were insufficient to allow PCR, only microscopic counting was performed. Both in liver (Fig. 5A) and spleen (Fig. 5B), paromomycin-R parasites reached the highest burdens while no significant differences were noted between WT and paromomycin-R parasites in the bone-marrow (Fig. 5C). As could be expected based on their *in vitro* metacyclogenesis profile, miltefosine-R parasites displayed significantly lower burdens in all target organs.

Intracellular amastigote stress resistance: While paromomycin-R parasites showed an enhanced tolerance towards nitrosative stress, no significant differences could be detected between miltefosine-R and WT-parasites (Fig. 6A). Measurement of the endogenous IL-10 production by infected macrophages upon stimulation with LPS and IFN-γ revealed a significant lower IL-10 production of miltefosine-R

infected macrophages, whereas no significant differences could be observed for paromomycin-R parasites compared to paromomycin-susceptible WT-parasites (Fig. 6B).

Discussion

Miltefosine and paromomycin were approved for the treatment of VL to combat the expanding antimony resistance in the Indian subcontinent, where their use in combination therapy is now being explored as first-line option to avoid development of miltefosine or paromomycin resistance. [22, 23] Experimental selection of paromomycin-resistant *L. donovani* and *L. infantum* strains was shown to occur fairly rapidly both *in vitro* and *in vivo* [5, 24] and resistant clinical isolates have already been described.[2] The number of miltefosine treatment failures in the Indian subcontinent has increased up to 20% within only one decade of use [1, 25] without any link between relapse and susceptibility to miltefosine [1, 26]. In *L. infantum*, a noticeable decrease in miltefosine susceptibility was demonstrated *in vitro* for relapse isolates in Brazil [4] and isolation of miltefosine-resistant isolates has been reported.[2, 3] Studying modifications associated with the resistant phenotype should provide valuable insights to monitor emergence of drug resistance in the field. One of the phenotypic traits that recently gained a lot of interest is parasite fitness, defined as the complex interaction of numerous factors guaranteeing survival, reproduction and transmission between hosts in a given environment.[27-29] While for most organisms the acquisition of drug resistance associates with several drawbacks, the effect of drug resistance in *Leishmania* on fitness remains fairly debatable and appears to be dependent on the respective drug and parasite species.[30-34] For antimony-resistant *L. donovani*, various reports suggested a trend towards increased fitness enhancing the spreading potential of resistant parasites.[7-9] Indian miltefosine relapse isolates displayed an enhanced *in vitro* metacyclogenesis and infectivity,[12] a promastigote-selected miltefosine-resistant *L. donovani* strain was shown to cope better with oxidative stress[35] and an experimentally promastigote-selected *L. major* isolate demonstrated an increased

metacyclogenesis profile.[36] However, most of these studies relied on large groups of non-matched isolates and such findings should be interpreted with caution as the *in vitro* comparison of dissimilar *Leishmania* species or strains can be obscured by various species/strain-dependent traits.[37] Ideally, resistance characteristics should be compared between the same pre- and post-resistance strain, but as few resistant clinical isolates are yet available, obtaining such couples may prove to be extremely challenging. Hence, experimental selection of drug resistance yet remains the only way to accommodate this need. Our research group developed *in vitro* [2, 5] and *in vivo* [24] selection protocols on the intracellular amastigote stage. Compared to paromomycin,[5] selection for miltefosine resistance proved to be much more complex with only one *L. infantum* isolate (LEM3323) gaining a definite miltefosine-resistant phenotype on both promastigote and amastigote level.[14] To assess the possible impact on fitness, LEM3323 and its miltefosine- and paromomycin-resistant derived counterparts were subjected to the same battery of 'virulence' assays. Although an increased parasite fitness for a promastigote-selected paromomycin-resistant strain has been described [13], no apparent benefit of paromomycin resistance could be demonstrated for the promastigote stage. When focusing on the intracellular amastigote stage, the paromomycin-resistant strain did show marked benefits over WT parasites, *e.g.* enhanced *in vitro* and *in vivo* amastigote replication and resistance towards macrophage-induced stress responses.

After comparison of the WT and miltefosine-resistant strain, all results suggested a decrease in parasite fitness upon acquisition of miltefosine resistance under the stated experimental conditions. This decline was evident on both the promastigote and the intracellular amastigote stage and was reflected by reduced replication potential, metacyclogenesis and stress tolerance. Although it was hypothesized that miltefosine treatment outcome could be related to the enhanced infectivity and metacyclogenesis of miltefosine relapse isolates [12], the present study does not support this. Indeed, if relapse isolates would in fact display an enhanced infectivity, experimental selection of drug resistance would be more straightforward. The association between high infectivity and facilitated resistance development may explain why our selection protocol failed for most *L. infantum* and *L. donovani* isolates, and was

successful only for the LEM3323 whose promastigotes were shown to cause massive macrophage infection and intracellular replication, even at an infection ratio of 1 promastigote/macrophage (unpublished results, Hendrickx S). Another natural miltefosine-resistant clinical isolate (LEM5159) [2] shared almost identical *in vitro* infectivity though its amastigote replication profile was less pronounced (unpublished results, Hendrickx S).

Although the fitness decrease in miltefosine-resistant *L. infantum* should be confirmed in other miltefosine-resistant isolates, additional research by our group already suggested a decline in fitness upon repeated miltefosine exposure of amastigotes *in vivo* as a reduced amastigote-to-promastigote transformation ability.[24] Despite the fact miltefosine resistance will certainly not be the sole factor contributing to the increasing levels of miltefosine treatment failures worldwide,[1, 4] it is encouraging to know that actual full-blown miltefosine resistance comes with a fitness cost for the parasite and may to some extent restrict extensive spread of primary resistance. With regard to the latter, parasite fitness in the vector should be explored as well. Finally, some pharmacokinetic and pharmacodynamic properties of miltefosine should encourage revision of the current miltefosine treatment regimen as drug exposure appears inadequate in some patients.[38]

Acknowledgements

The authors want to acknowledge Dr. Gaëlle Boulet for the skillful design of the PCR primers and the statistical analysis. Mandy Vermont and Pim-Bart Feijens are acknowledged for running the *in vivo* experiments.

Funding

This work was funded by the Research Fund Flanders (FWO: project G051812N). LMPH is a partner of the Antwerp Drug Discovery Network (ADDN, www.addn.be).

Transparency declarations

None to declare.

References

1. Rijal S, Ostyn B, Uranw S, et al. Increasing failure of miltefosine in the treatment of kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. *Clin Infect Dis* 2013; **56**: 1530-8.

2. Hendrickx S, Boulet G, Mondelaers A, et al. Experimental selection of paromomycin and miltefosine resistance in intracellular amastigotes of Leishmania donovani and L. infantum. *Parasitol Res* 2014; **113**: 1875-81.

3. Cojean S, Houze S, Haouchine D, et al. Leishmania resistance to miltefosine associated with genetic marker. *Emerg Infect Dis* 2012; **18**: 704-6.

4. Carnielli JB, de Andrade HM, Pires SF, et al. Proteomic analysis of the soluble proteomes of miltefosine-sensitive and -resistant Leishmania infantum chagasi isolates obtained from Brazilian patients with different treatment outcomes. *J Proteomics* 2014; **108**: 198-208.

5. Hendrickx S, Inocencio da Luz RA, Bhandari V, et al. Experimental induction of paromomycin resistance in antimony-resistant strains of L. donovani: outcome dependent on in vitro selection protocol. *PLoS Negl Trop Dis* 2012; **6**: e1664.

6. Guha R, Das S, Ghosh J, et al. Antimony resistant Leishmania donovani but not sensitive ones drives greater frequency of potent T-regulatory cells upon interaction with human PBMCs: role of IL-10 and TGF-beta in early immune response. *PLoS Negl Trop Dis* 2014; **8**: e2995.

7. Vanaerschot M, Maes I, Ouakad M, et al. Linking in vitro and in vivo survival of clinical Leishmania donovani strains. *PLoS One* 2010; **5**: e12211.

8. Vanaerschot M, De DS, Rijal S, et al. Antimonial resistance in Leishmania donovani is associated with increased in vivo parasite burden. *PLoS One* 2011; **6**: e23120.

9. Ouakad M, Vanaerschot M, Rijal S, et al. Increased metacyclogenesis of antimony-resistant Leishmania donovani clinical lines. *Parasitology* 2011; **138**: 1392-9.

10. Mukherjee B, Mukhopadhyay R, Bannerjee B, et al. Antimony-resistant but not antimony-sensitive Leishmania donovani up-regulates host IL-10 to overexpress multidrug-resistant protein 1. *Proc Natl Acad Sci U S A* 2013; **110**: E575-E582.

11. Mukhopadhyay R, Mukherjee S, Mukherjee B, et al. Characterisation of antimony-resistant Leishmania donovani isolates: biochemical and biophysical studies and interaction with host cells. *Int J Parasitol* 2011; **41**: 1311-21.

12. Rai K, Cuypers B, Bhattarai NR, et al. Relapse after treatment with miltefosine for visceral leishmaniasis is associated with increased infectivity of the infecting Leishmania donovani strain. *MBio* 2013; **4**: e00611-e00613.

13. Bhandari V, Sundar S, Dujardin JC, et al. Elucidation of cellular mechanisms involved in experimental paromomycin resistance in Leishmania donovani. *Antimicrob Agents Chemother* 2014; **58**: 2580-5.

14. Hendrickx S, Mondelaers A, Eberhardt E, et al. Intracellular amastigote replication may not be required for successful in vitro selection of miltefosine resistance in Leishmania infantum. *Parasitol Res* 2015; **114**: 2561-5.

15. Vermeersch M, Inocencio da Luz RA, Tote K, et al. In vitro susceptibilities of Leishmania donovani promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. *Antimicrob Agents Chemother* 2009; **53**: 3855-9.

16. da Silva R, Sacks DL. Metacyclogenesis is a major determinant of Leishmania promastigote virulence and attenuation. *Infect Immun* 1987; **55**: 2802-6.

17. Kerstens M, Boulet G, Pintelon I, et al. Quantification of Candida albicans by flow cytometry using TO-PRO((R))-3 iodide as a single-stain viability dye. *J Microbiol Methods* 2013; **92**: 189-91.

18. Inocencio da Luz RA, Vermeersch M, Dujardin JC, et al. In vitro sensitivity testing of Leishmania clinical field isolates: preconditioning of promastigotes enhances infectivity for macrophage host cells. *Antimicrob Agents Chemother* 2009; **53**: 5197-203.

19. Stauber L. Leishmaniasis in the hamster. In: W.H.Cole, ed. *Some Physiological Aspects and Consequences of Parasitism*. New Brunswisck, New Jersey: Rutgers University Press, 1955; 76-90.

20. Carter KC, Hutchison S, Boitelle A, et al. Sodium stibogluconate resistance in Leishmania donovani correlates with greater tolerance to macrophage antileishmanial responses and trivalent antimony therapy. *Parasitology* 2005; **131**: 747-57.

21. Deschacht M, Van AT, Hendrickx S, et al. Role of oxidative stress and apoptosis in the cellular response of murine macrophages upon Leishmania infection. *Parasitology* 2012; **139**: 1429-37.

22. Davidson RN, den Boer M, Ritmeijer K. Paromomycin. *Trans R Soc Trop Med Hyg* 2009; **103**: 653-60.

23. Dhillon GP, Sharma SN, Nair B. Kala-azar elimination programme in India. *J Indian Med Assoc* 2008; **106**: 664, 666-4, 668.

24. Hendrickx S, Mondelaers A, Eberhardt E, et al. In Vivo Selection of Paromomycin and Miltefosine Resistance in Leishmania donovani and L. infantum in a Syrian Hamster Model. *Antimicrob Agents Chemother* 2015; **59**: 4714-8.

25. Sundar S, Singh A, Rai M, et al. Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin Infect Dis* 2012; **55**: 543-50.

26. Bhandari V, Kulshrestha A, Deep DK, et al. Drug susceptibility in Leishmania isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. *PLoS Negl Trop Dis* 2012; **6**: e1657.

27. Borrell S, Gagneux S. Infectiousness, reproductive fitness and evolution of drug-resistant Mycobacterium tuberculosis. *Int J Tuberc Lung Dis* 2009; **13**: 1456-66.

28. Orr HA. Fitness and its role in evolutionary genetics. *Nat Rev Genet* 2009; **10**: 531-9.

29. Natera S, Machuca C, Padron-Nieves M, et al. Leishmania spp.: proficiency of drug-resistant parasites. *Int J Antimicrob Agents* 2007; **29**: 637-42.

30. Vanaerschot M, Decuypere S, Berg M, et al. Drug-resistant microorganisms with a higher fitness - can medicines boost pathogens? *Crit Rev Microbiol* 2012; **39**: 384-94.

31. Detke S, Chaudhuri G, Kink JA, et al. DNA amplification in tunicamycin-resistant Leishmania mexicana. Multicopies of a single 63-kilobase supercoiled molecule and their expression. *J Biol Chem* 1988; **263**: 3418-24.

32. Kink JA, Chang KP. Biological and biochemical characterization of tunicamycin-resistant Leishmania mexicana: mechanism of drug resistance and virulence. *Infect Immun* 1987; **55**: 1692-700.

33. Al-Mohammed HI, Chance ML, Bates PA. Production and characterization of stable amphotericin-resistant amastigotes and promastigotes of Leishmania mexicana. *Antimicrob Agents Chemother* 2005; **49**: 3274-80.

34. Gazola KC, Ferreira AV, Anacleto C, et al. Cell surface carbohydrates and in vivo infectivity of glucantime-sensitive and resistant Leishmania (Viannia) guyjanensis cell lines. *Parasitol Res* 2001; **87**: 935-40.

35. Mishra J, Singh S. Miltefosine resistance in Leishmania donovani involves suppression of oxidative stress-induced programmed cell death. *Exp Parasitol* 2013; **135**: 397-406.

36. Turner KG, Vacchina P, Robles-Murguia M, et al. Fitness and Phenotypic Characterization of Miltefosine-Resistant Leishmania major. *PLoS Negl Trop Dis* 2015; **9**: e0003948.

37. Dey T, Afrin F, Anam K, et al. Infectivity and virulence of Leishmania donovani promastigotes: a role for media, source, and strain of parasite. *J Eukaryot Microbiol* 2002; **49**: 270-4.

38. Dorlo TP, Rijal S, Ostyn B, et al. Failure of Miltefosine in Visceral Leishmaniasis Is Associated With Low Drug Exposure. *J Infect Dis* 2014.

Tables

	Intracellular amastigote susceptibility				Promastigote susceptibility			
Strain	**MIL (µM)**		**PMM (µM)**		**MIL (µM)**		**PMM (µM)**	
	IC_{50}	**± SEM**	**IC_{50}**	**± SEM**	**IC_{50}**	**± SEM**	**IC_{50}**	**± SEM**
WT	2.3	± 0.5	89.3	± 4.5	5.3	± 0.3	129.8	± 11.3
MIL	>20		78.5	± 9.2	>40		177.8	± 29.7
PMM	0.5	± 0.1	212.6	± 31.0	8.2	± 1.1	138.1	± 6.0

Table 1: Promastigote and intracellular amastigote susceptibilities of the wild-type (WT), miltefosine-resistant (MIL) and paromomycin-resistant (PMM) strain. For promastigote susceptibility, 10^5 log-phase promastigotes were exposed to serial dilutions of miltefosine or paromomycin for 72h at 25°C. Parasite multiplication was assessed after addition of resazurin and fluorimetric reading. For amastigote susceptibility, primary peritoneal mouse macrophages were infected with metacyclic promastigotes at an infection ratio of 15 stages per cell. Total parasite burdens were microscopically assessed on Giemsa-stained wells after 5 days of incubation. The results are expressed as percentages reduction in parasite burden compared to untreated infected controls and IC_{50}s were calculated. Susceptibility values are the result of at least three independent replicates and are expressed as the mean IC_{50}-value ± standard error of mean (SEM).

Figures

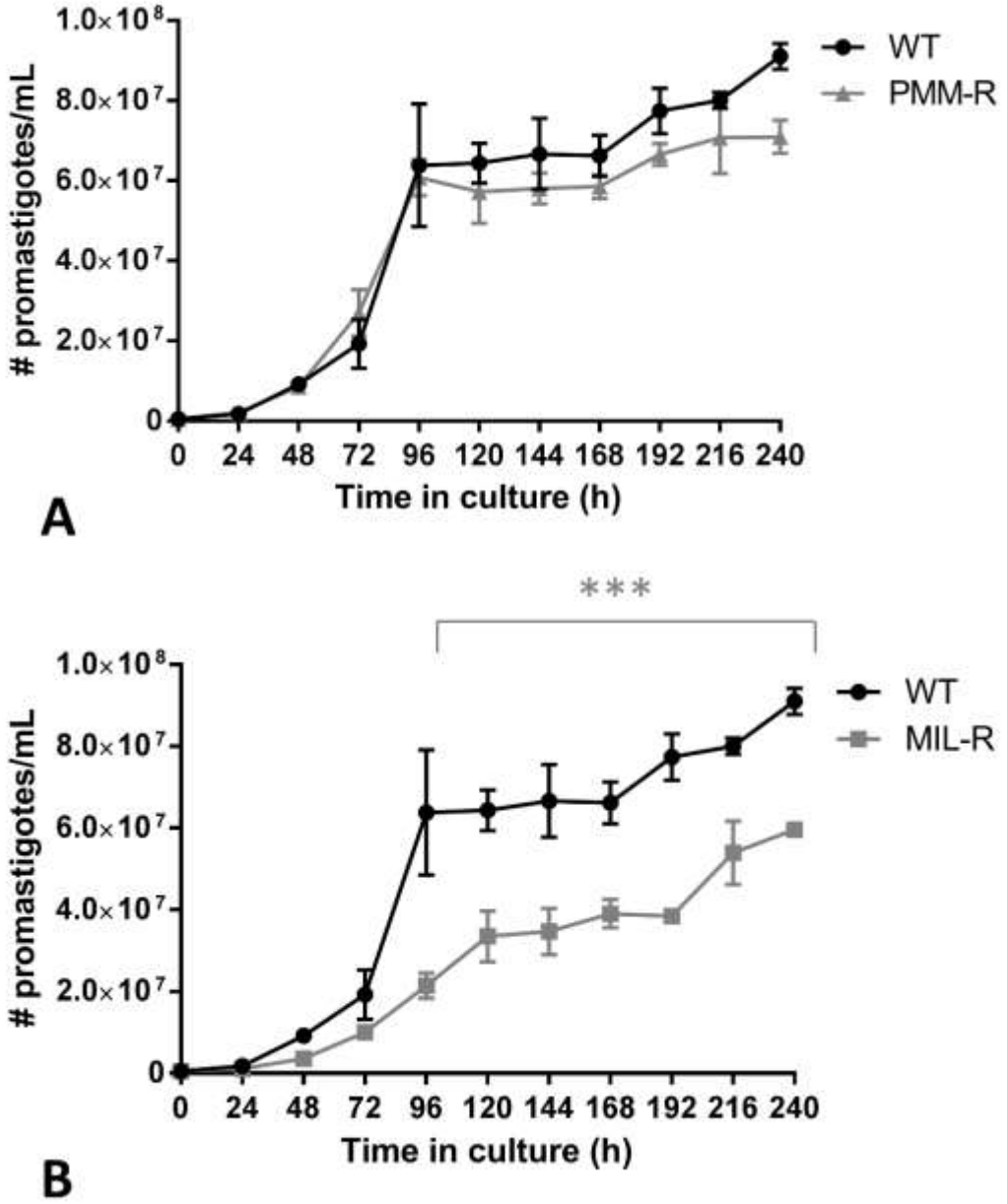
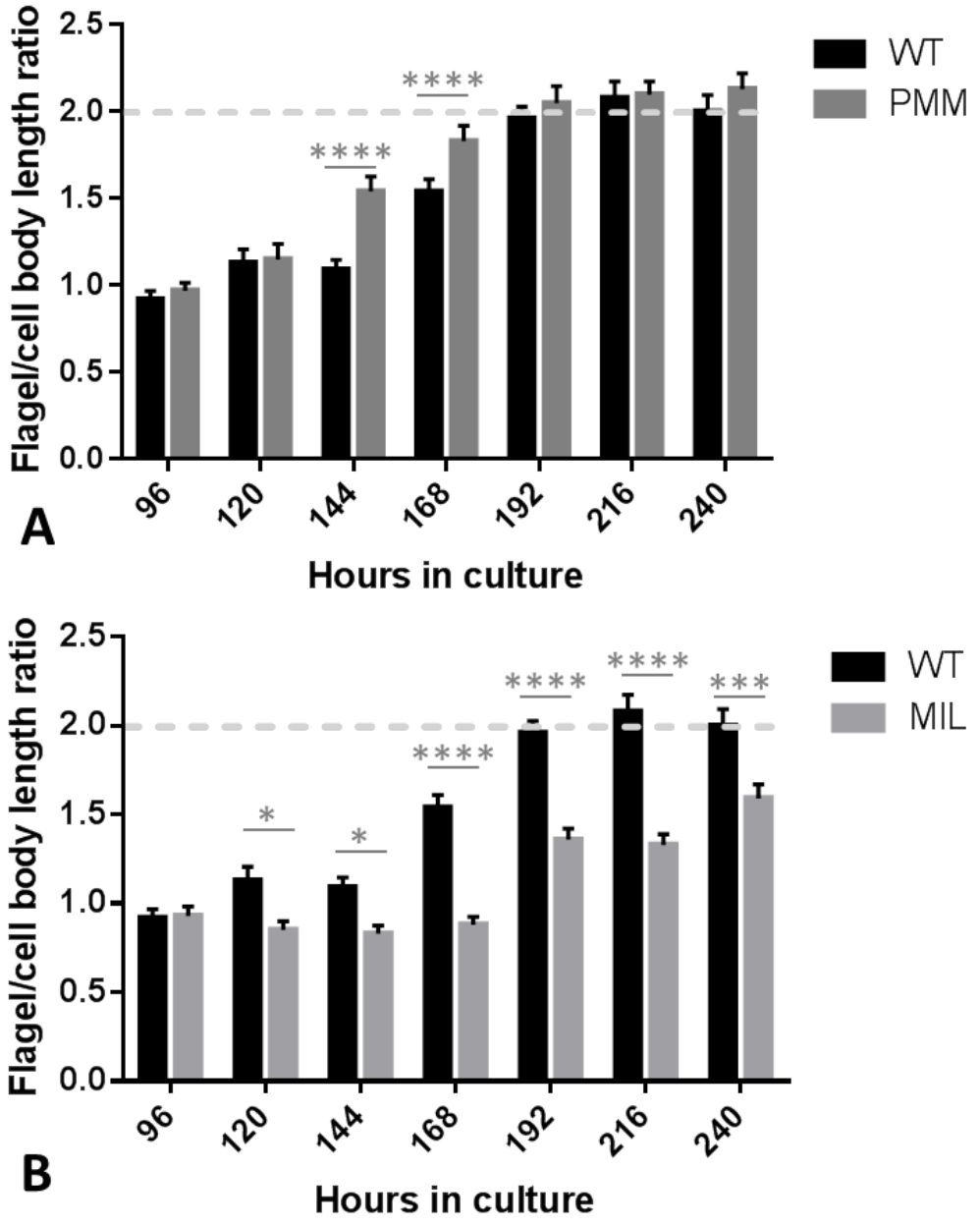
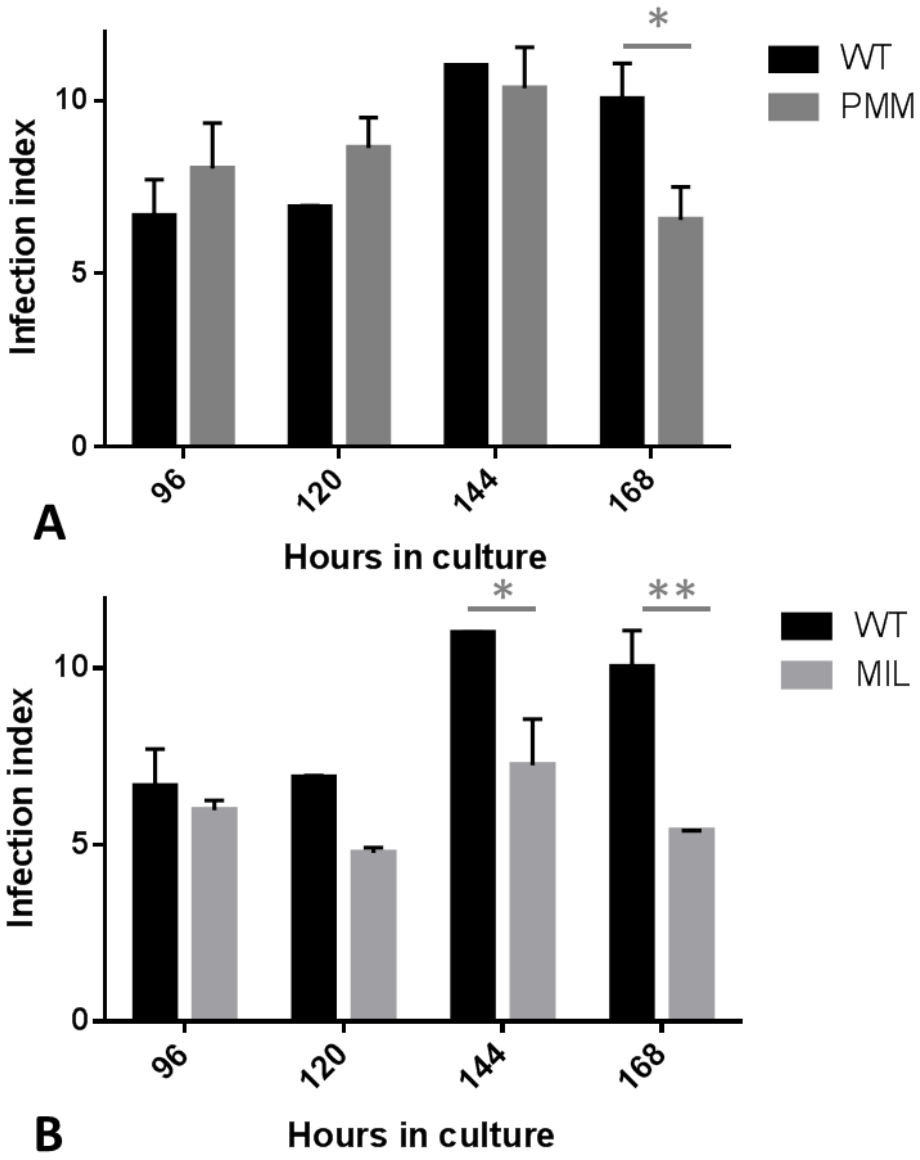


Figure 1: Promastigote growth curves of wild-type (WT), paromomycin-resistant (PMM-R) and miltefosine-resistant (MIL-R) matched isolates. A/ WT and PMM-R parasites show comparable growth; **B/** MIL-R parasites show significantly decreased growth compared to WT starting at 96h in culture ($p<0.001$). Results are expressed as mean ± standard error of mean (SEM) and are based on three independent replicates run in duplicate.



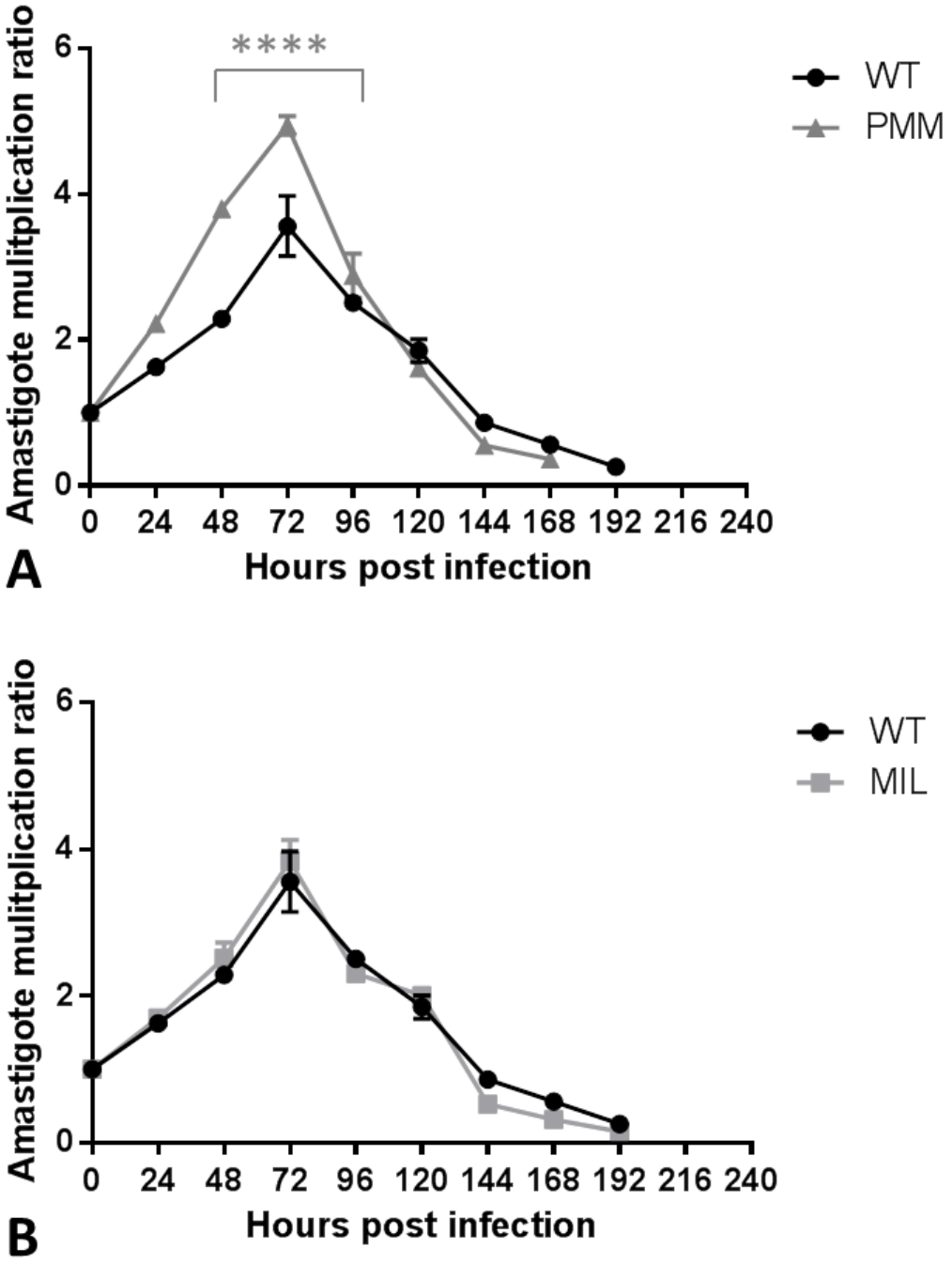
384

385 **Figure 2: Metacyclogenesis of wild-type (WT), paromomycin-resistant (PMM) and miltefosine-resistant (MIL) matched**
386 **isolates.** Promastigotes were labeled metacyclic when the flagellum/cell body length ratio exceeded the preset cut-off 2 (grey
387 dotted line). **A/** WT and PMM parasites demonstrated significant changes at 144h and 168h; **B/** WT and MIL parasites revealed
388 significant morphological differences at all selected time points apart from 96h. Results are expressed as mean ± standard error
389 of mean (SEM) and are based on three independent replicates measuring the flagel/cell body length ratio of minimum 50
390 promastigotes (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$).



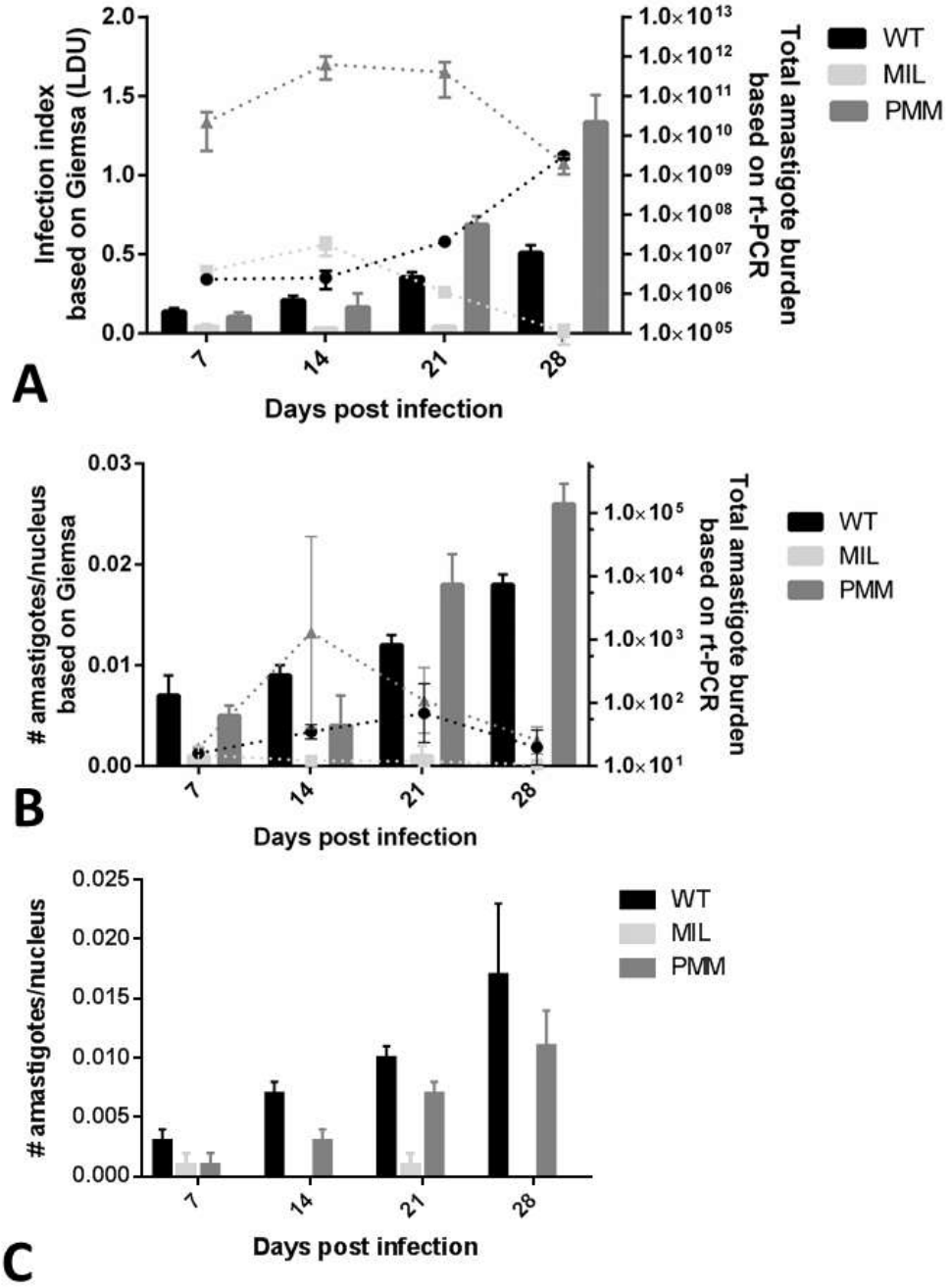
391

392 **Figure 3: Promastigote infectivity for wild-type (WT), paromomycin-resistant (PMM) and miltefosine-resistant (MIL) matched**
393 **isolates.** For both strains, the highest infection ratio was obtained at 144h. **A/** Infection indices of WT and PMM parasites differ
394 significantly at 168h; **B/** Although the mean infection index of MIL promastigotes was maximal at 144h, infectivity of MIL
395 promastigotes was significantly lower at 144h and 168h compared to WT parasites. Results are expressed as mean ± standard
396 error of mean (SEM) and are based on three independent replicates run in duplicate (* $p<0.05$; ** $p<0.01$).



397

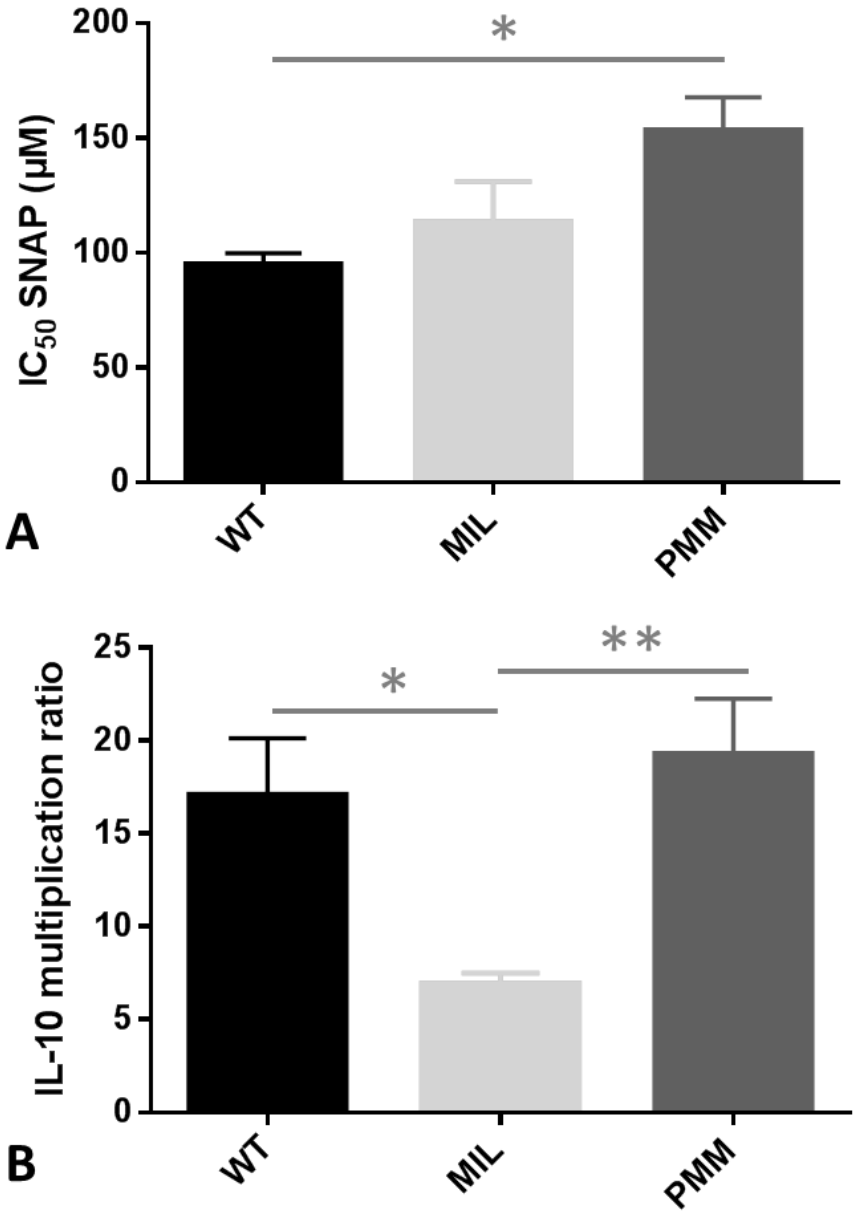
398 **Figure 4: *In vitro* amastigote growth curves of wild-type (WT), paromomycin-resistant (PMM) and miltefosine-resistant (MIL)**
399 **matched isolates. A/** Comparison of WT and PMM parasites reveals a significantly enhanced amastigote growth for PMM
400 parasites between 48h and 96h post infection; **B/** no significant differences could be observed for WT and MIL parasites
401 ($p>0.0001$). Results are expressed as mean ± standard error of mean (SEM) and are based on three independent replicates run
402 in duplicate.



403

404 **Figure 5:** ***In vivo*** **amastigote growth of wild-type (WT), paromomycin-resistant (PMM) and miltefosine-resistant (MIL)**
405 **matched isolates in liver (A), spleen (B) and bone-marrow (C) of infected Balb/c mice.** Results are expressed as mean ±
406 standard deviation and are based on three independent replicates. The bar graphs represent the infection indices based on
407 microscopic counting of Giemsa-stained smears and are expressed either as Leishman-Donovan units for the liver or as the
408 average number of amastigotes per nucleus when infection was limited in spleen and bone-marrow. The dotted line graphs
409 represent the amastigote burden as determined by rt-PCR.

410



411

412 **Figure 6: Tolerance of amastigotes of wild-type (WT), paromomycin-resistant (PMM) and miltefosine-resistant (MIL) matched**
413 **isolates to cellular stress responses. A/** Tolerance to nitrosative stress upon S-nitroso-N-acetyl-DL-penicillamine (SNAP)
414 exposure. No significant differences could be observed between WT and MIL parasites, while PMM parasites showed a
415 significantly enhanced tolerance towards nitrosative stress inside primary peritoneal mouse macrophages ($p<0.05$). **B/**
416 Endogenous IL-10 production of infected cells stimulated with LPS and IFN-γ. Statistical differences between MIL and WT
417 parasites were noted (* $p<0.05$), whereas IL-10 production of WT and PMM strains did not statistically differ. Results are
418 expressed as mean ± standard error of mean (SEM) and are based on four independent replicates run in duplicate (** $p<0.01$).

419