Targeted Genetic Knockouts in *Leishmania mexicana* Reveal Roles for Sphingolipid Metabolism in Drug Sensitivity

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Abstract Leishmaniasis, caused by the eukaryotic pathogen *Leishmania spp.*, is a disease for which historical neglect has left us with a limited armoury of therapeutics. Emerging issues with the few drugs we do have present an increasing risk to global health. There is a pressing need for novel anti-leishmanials and a comprehensive understanding of their mode of action. Previous study has implicated the central role of lipids in the modes of action of both amphotericin B and miltefosine, two drugs used in treatment of leishmaniasis. To investigate the modes of action of these drugs in the context of lipid metabolism, a curated library of CRISPR/Cas9 modified *Leishmania mexicana* with genetic knockout of key genes in lipid metabolism were subjected to drug sensitivity and phenotypic screening. This library screening approach uncovered genes for which knockout contributes to decreased membrane integrity and resultant sensitivity to drug pressure. The most significant changes in drug sensitivity were found after knockout of inositol phosphoryl ceramide synthase, a key enzyme in sphingolipid synthesis, where a 4-fold increase in resistance to miltefosine was observed, implicating the enzyme or it's associated metabolites in the mode of action.

Introduction

Leishmaniasis is treated with the compounds Amphotericin B (AmB) and miltefosine (MTF)[1]. AmB acts primarily through sequestering of ergosterol in cell membranes leading to pore formation and lysis[2], but a complete understanding of the mode of action (MoA) of MTF has proven elusive. A role for sphingolipids (SLs) in MTF MoA is one of many areas implicated[3]. SLs, a diverse class of amphipathic lipids with a core 3-domain structure[4] (Fig. 1A), are found both in *Leishmania* and their mammalian hosts, with some key differences. SL synthesis can be viewed in 3 steps (Fig. 1B), with parasite/host differences mainly arising in the head group addition stage - *Leishmania* make inositol phosphoryl-ceramide (IPC) and mammals sphingomyelin[5]. Here, we show a link between drug MoA and Serine Palmitoyl Transferase (SPT), Ceramide Synthase (CerS) and IPC Synthase (IPCS), three key enzymes in *Leishmania* SL synthesis (Fig. 1B).

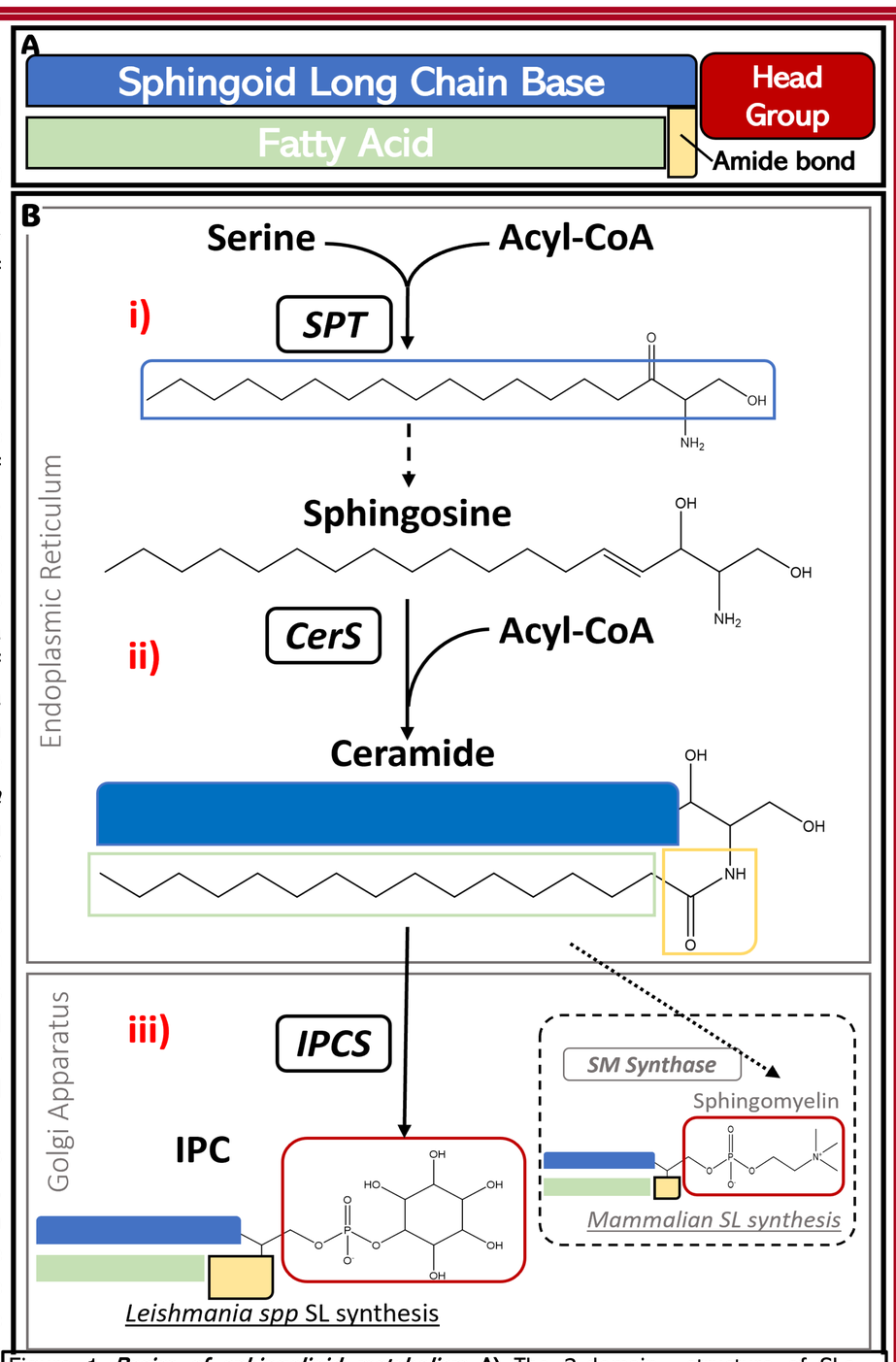

Figure 1 ***Basics of sphingolipid metabolism*** **A)** The 3-domain structure of SLs – hydrophobic sphingoid long chain base (blue), charged head group (red), fatty acid chain (green) linked by an amide bond (yellow) **B)** Key steps of sphingolipid metabolism: i) condensation of serine and acyl-CoA by SPT; ii) N-acylation of sphingosine by CerS; iii) addition of head group by SL synthases, highlighting *Leishmania*/mammal differences.

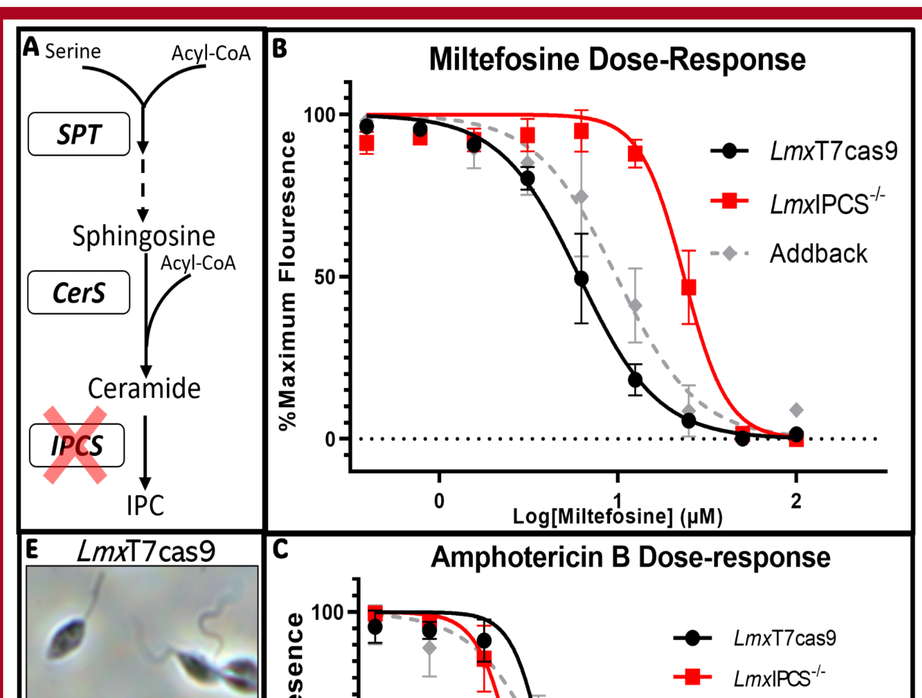

Figure 3 ***IPCS KOs show specific MTF resistance*** **A)** IPCS KO in SL synthesis. **B) & C)** Parental, KO and AB response to MTF and AmB **D)** Parental and KO HOS response **E)** Parental and KO morphology (fixed)

Results

KO of IPCS gene in *L. mex.* (Fig. 3A) yielded a KO (*Lmx*IPCS$^{-/-}$) with 4-fold resistance to MTF (Fig. 3B) compared to parental line (*Lmx*T7cas9) and hyper-sensitivity to AmB. Restoration of IPCS (Addback, AB) restored drug sensitivity towards parental levels. HOS assay showed no evidence of deficiency (Fig. 3D), though an abnormal rounded morphology was observed. (Fig. 3E). Full KO of the SPT catalytic subunit LCB2 is only possible with additional compensatory KOs[8]. Three double gene KOs – LCB2 deleted with: CerS (*Lmx*ΔLCB2/ΔCerS); an ABC transporter (*Lmx*ΔLCB2/ΔABC3A); or a hypothetical protein (*Lmx*ΔLCB2/ΔLmxM.13.1540) – display two distinct phenotypes. *Lmx*ΔLCB2/ΔCerS and *Lmx*ΔLCB2/ΔABC3A show hyper-sensitivity to MTF and AmB (Fig. 4A&B), rounded morphology (Fig. 4C), and membrane deficiency (Fig. 4D). *Lmx*ΔLCB2/ΔLmxM.13.1540 shows MTF resistance, no change in AmB sensitivity, normal HOS response and standard cell morphology.

Methods

Generation of a lipid metabolism knockout library A knockout (KO) library was generated in *Leishmania mexicana* utilising LeishGEdit protocol[6]. **Drug Sensitivity Assays** EC_{50} of antileishmanial compounds was determined using a standard fluorescence-based in vitro proliferation assay with serial dilutions of compounds[7]. Normalised fluorescence data was plotted in GraphPad Prism. **Hypo-osmotic Shock Assays** Cell recovery after Hypo-osmotic Shock (HOS) was measured by monitoring absorbance at 550nm for 5 minutes after addition of water to cell suspensions. Decrease in absorbance corresponds to an increase in cell volume (Fig. 2).

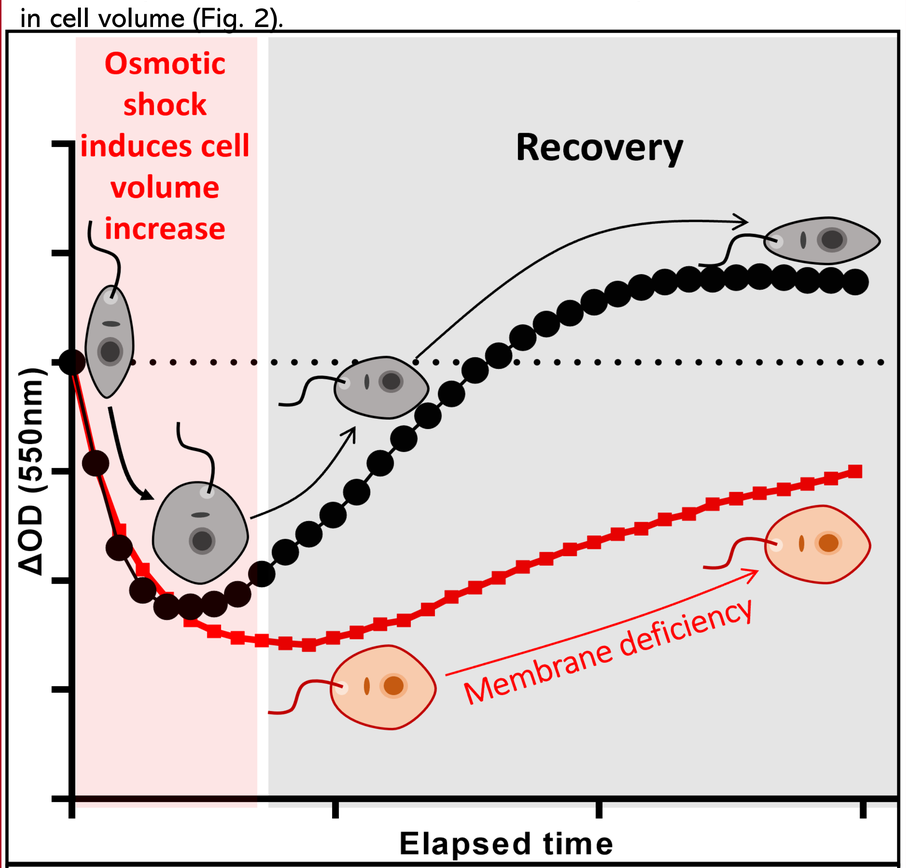

Figure 2 ***HOS assays to investigate cell membrane integrity*** Change in optical density at 550nm can be used to monitor cell volume after hypo osmotic shock to evaluate cell membrane integrity.

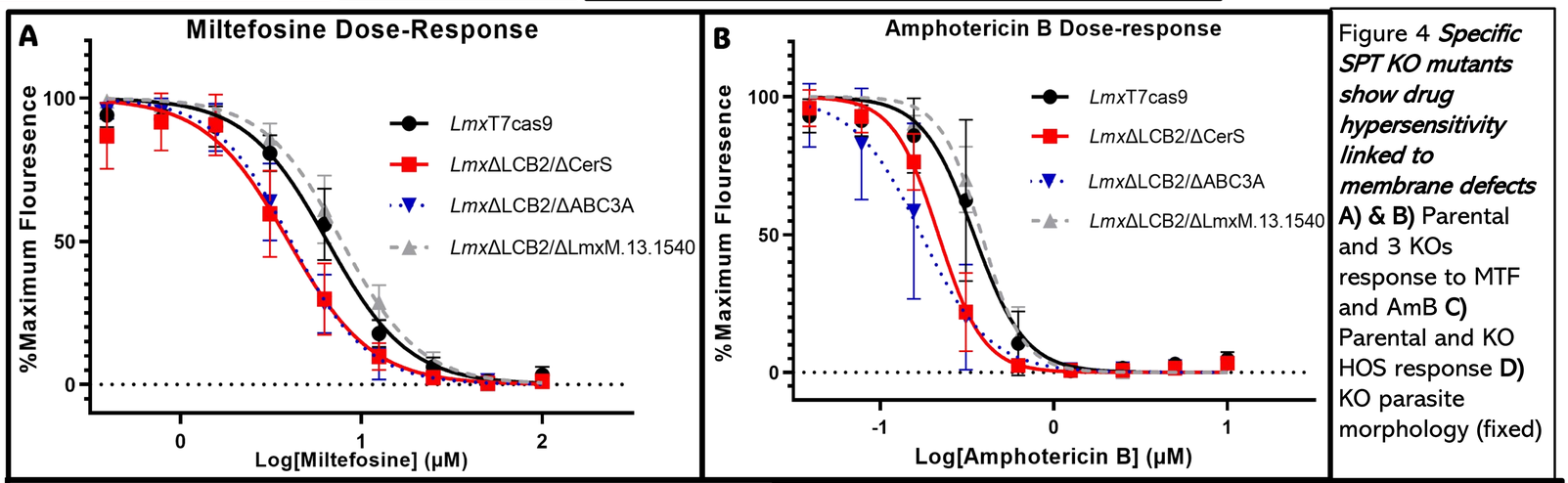

Figure 4 ***Specific SPT KO mutants show drug hypersensitivity linked to membrane defects*** **A) & B)** Parental and 3 KOs response to MTF and AmB **C)** Parental and KO HOS response **D)** KO parasite morphology (fixed)

Discussion

Using SL synthesis KOs in *L. mexicana*, we have shown a link between SLs and the MoA of two anti-leishmanials. The resistance of IPCS KOs to MTF but not AmB separates the drug MoAs and links MTF MoA specifically to IPCS/IPC. The converse hyper-sensitivity of SPT KOs to MTF appears to result from a membrane deficiency, supported by an inability to recover from HOS (Fig. 4C), suggesting global ablation of SL synthesis at the first step impairs membrane integrity and fluidity, while deletion of IPCS negates a more direct interaction between MTF and IPC. The hyper-sensitivity to AmB in SPT and IPCS KOs is consistent with evidence that SLs shield *Leishmania* against the cytotoxic effects of sterol-binding compounds[9]. KO of a hypothetical protein LmxM.13.1540 reverses the ΔSPT phenotype, with MTF resistance and no membrane deficiency observed. A potential explanation may involve an association through genomic location of LmxM.13.1540 to the phospholipid translocase Miltefosine Transporter[8][10], though a full functional analysis of this gene would be necessary to confirm this. The results highlighted contribute to a fuller understanding of MTF MoA and provide proof of concept for the use of this KO library in investigating novel anti-leishmanial MoA.

References 1. Mathison BA, Bradley BT, *Laboratory Medicine*, **2022**, 54(4); 2. Baginski M, Czub J, *Current Drug Metabolism*, **2009,** 10(5); 3. Armitage EG et al, *Antimicrobial Agents and Chemotherapy*, **2018,** 62(5); 4. Futerman AH. Chapter 9 - Sphingolipids. In: *Biochemistry of Lipids, Lipoproteins and Membranes*. 7th Edition ed. Elsevier; **2021**; 5. Mina JG, Denny PW, *Parasitology*, **2017**, 145(2); 6. Beneke T, Gluenz E, *Methods in Molecular Biology*, **2019,** 189–210; **7.** Chadbourne FL et al, *Journal of Peptide Science*, **2011**, 17(11); 8. Alpizar-Sosa EA et al, *Frontiers in Cellular and Infection Microbiology*, **2022**, 12; 9. Haram CS et al, *Journal of Biological Chemistry*, **2023**, 299(6); 10. Pérez-Victoria FJ et al, *Journal of Biological Chemistry*, **2003**, 278(50)

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