Abstract

Leishmaniasis continues to be one of the most devastating yet neglected tropical diseases. Among the other forms, visceral leishmaniasis (VL), also known as kala azar, is associated with higher suffering and mortality rates, over 95% if no proper treatment is given. Therefore, it poses a significant health concern to people, particularly those of low socio-economic status in developing countries like Brazil, East Africa, and India. Despite decades of research efforts, current antileishmanial therapies suffer from serious limitations, including drug resistance, toxicity, and the absence of effective vaccines. The causative agent, *Leishmania donovani*, a protozoan parasite of the *Trypanosomatidae* family, alternates between motile promastigote and non-motile amastigote forms, primarily targeting macrophages in visceral organs such as the spleen, liver, bone marrow, and lymph nodes.

Leishmania parasites employ different immune evasion mechanisms to survive inside the host macrophages, notably by neutralizing oxidative stress within the macrophages. Different Reactive oxygen species (ROS) are generated as key components of the immune response within the phagolysosomal environment of macrophages. *Leishmania* effectively neutralizes these ROS by the unique trypanothione-based redox system, distinct from the host's antioxidant mechanisms, rendering it an attractive target for therapeutic intervention. Several enzymes are involved in the biosynthesis of trypanothione, among which glutathione synthetase (GS) plays a critical and indispensable role in maintaining redox equilibrium. The significant structural differences between human and parasitic glutathione synthetase present an opportunity for developing selective inhibitors targeting leishmanial glutathione synthetase, thereby enabling a rational development of novel antileishmanial agents. However, before compound screening or development, it is essential to validate the indispensability of the glutathione synthetase enzyme for the parasite.

In this study, we employed CRISPR-Cas9 genome editing to generate *Leishmania donovani* strains deficient in the GS gene. These GS-deficient parasites exhibited severely impaired growth and reduced infectivity in macrophages, highlighting the enzyme's essential role in parasite survival and pathogenicity. To further explore GS as a drug target, we constructed a three-dimensional homology model of the *L. donovani* GS enzyme and performed virtual screening of FDA-approved zinc compounds. Of the

candidates, four compounds showed promising binding affinity in molecular docking and dynamics simulations.

Subsequent in vitro assays confirmed the potent antileishmanial activity of these compounds at low concentrations. Enzyme inhibition studies using recombinant GS expressed in *E. coli* verified that both compounds significantly reduced enzyme activity, supporting their role as direct inhibitors. Flow cytometric analyses revealed that treatment with valrubicin and ciclesonide induced hallmark features of apoptosis-like cell death in *Leishmania*, including morphological alterations, increased intracellular reactive oxygen species (ROS), G0/G1 cell cycle arrest, DNA fragmentation, and phosphatidylserine externalization.

Together, our findings establish glutathione synthetase as a vital enzyme for *Leishmania* viability and pathogenesis and identify valrubicin and ciclesonide as potent inhibitors that disrupt redox homeostasis, leading to programmed cell death. This study not only deepens our understanding of redox biology in *Leishmania* but also presents a strong case for repurposing these compounds as novel therapeutic candidates for the treatment of visceral leishmaniasis.

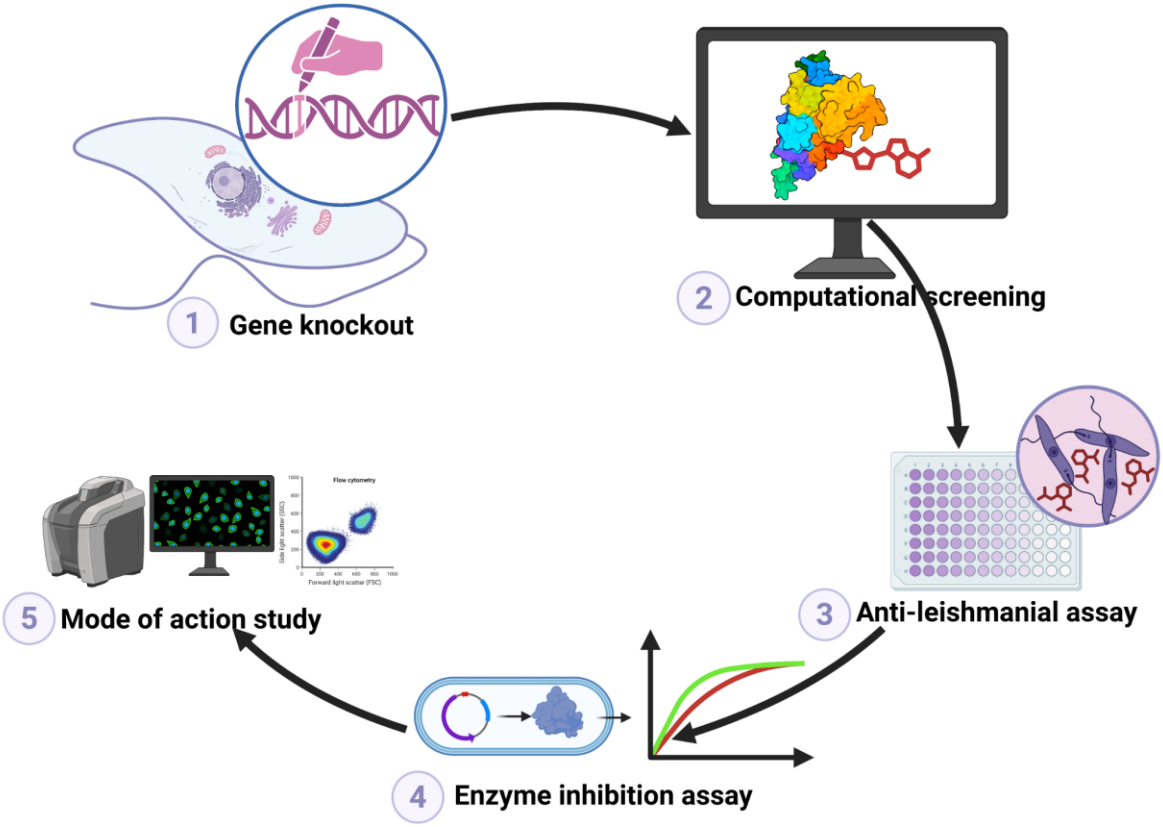

Figure: Graphical abstract of the study. The workflow starts with the genetic validation of drug targets in *Leishmania donovani* parasites through CRISPR-Cas9-mediated gene editing, followed by computational screening of potential inhibitors against the validated drug targets. *In vitro* evaluation of the selected inhibitors on the parasites, in vitro enzyme-inhibition assay, and investigation of the mechanism of parasitic death by the inhibitors.