Biochemical and Computational Studies on Drug Target Enzyme of *Leishmania*.

A thesis

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by

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Chapter 6

Conclusion & Future Scope

Visceral leishmaniasis (VL), one of the most lethal neglected tropical diseases, is still a public health challenge due to the limitations of current therapeutics. *Leishmania* parasites exhibit remarkable adaptability and drug resistance due to their unique biology. These traits make the management and treatment of leishmaniasis a significant global health challenge. The parasite's digenetic life cycle alternates between the sandfly vector and the mammalian host. In the sandfly, they exist as promastigotes, while in mammals, they transform into amastigotes that reside within macrophages. This dual-stage development demands extraordinary flexibility to survive hostile environments such as the insect gut and the innate immune defenses of the host. *Leishmania* possesses a unique thiol-based redox system centered on trypanothione, enhancing its tolerance to oxidative stress, both from drugs and the host immune system. This is pivotal for survival within macrophages and contributes to drug resistance. Understanding the vulnerabilities of the parasite's biology, such as metabolic pathways or essential proteins, we can design new drugs that are more

effective and less prone to resistance. This thesis aimed to address the gaps between existing knowledge about the parasite's vulnerabilities and novel therapeutic strategies by validating novel redox metabolism-related targets in *Leishmania* parasites. We specifically focused on glutathione synthetase (GS) enzyme, evaluating its drugability and identifying potential inhibitors from the FDA-approved drug library.

The central hypothesis of the study was that the redox balance in the *Leishmania* parasites can be effectively disrupted by inhibiting glutathione synthetase (GS) enzyme of the trypanothione-biosynthesis pathway. This disruption could induce programmed cell death in the parasites. Through a multifaceted approach, which includes gene knockout using the CRISPR-Cas9 technique, structure-based inhibitor screening, biochemical and cellular assays, we have ultimately achieved the following summary of the objectives-

Summary of the objectives and Hypotheses:

1. **Glutathione synthetase is critical for *Leishmania* parasites:** Although complete deletion of the glutathione synthetase gene could not be achieved, its partial disruption significantly impaired parasite growth and in vitro infectivity, highlighting its essential role and potential as a drug target. The replacement of the gene at two loci with neomycin- and puromycin-resistance cassettes likely reduced its gene dosage, which may have contributed to the observed slower growth and diminished capacity to infect macrophage cells.
2. **Computational drug repurposing identified FDA-approved compounds:** Five FDA-approved zinc-containing compounds were identified based on their selective binding to the active site residues of the parasitic glutathione synthetase enzyme in molecular docking studies, and their stable interactions

were further supported by molecular dynamics simulations. These compounds exhibited high binding affinities toward the parasitic enzyme, while demonstrating markedly lower affinities for the human homolog, indicating a strong degree of selectivity.

3. **Experimental evaluation:** The identified compounds were experimentally evaluated, and two of the five, valrubicin and ciclesonide, demonstrated significant anti-leishmanial activity at low concentrations, indicating their potential as effective therapeutic agents. However, both compounds also showed moderate cytotoxicity toward host cells in the in vitro assays.
4. **Biochemical assays and mode of parasitic death:** The compound valrubicin and ciclesonide could inhibit the glutathione synthetase enzyme that leads to induction of oxidative stress, cell cycle inhibition, DNA damage and an apoptosis-like death of the parasites.

In this study, the genetic validation and biochemical inhibition by the computationally identified compounds together establish glutathione synthetase in *Leishmania donovani* as a viable therapeutic target, providing a strong platform for rationale drug discovery. This study also suggests further preclinical evaluation of valrubicin and ciclesonide as novel anti-leishmanial agents.

Scientific and Therapeutic Implications

This study highlights the important role of glutathione synthetase (GS) in the survival and infectivity of *Leishmania donovani*. Although complete deletion of the GS gene could not be achieved, partial disruption led to reduced parasite growth and impaired

ability to infect host cells, suggesting that GS is essential for the parasite and can be considered a promising drug target. Computational analysis identified five FDA-approved zinc compounds that showed strong and selective binding to the parasitic GS enzyme, with minimal interaction with the human counterpart. Among them, two compounds, valrubicin and ciclesonide demonstrated significant anti-leishmanial activity at low concentrations during in vitro testing, along with moderate host cell toxicity. These findings not only contribute to our understanding of redox-related survival mechanisms in *Leishmania* but also offer potential leads for repurposing existing drugs to develop more effective and selective therapies against leishmaniasis.

Future Scope

The promising findings from the study give us several avenues for future research and translational ideas. These include-

1. **Stage-specific study of the glutathione synthetase role in the parasite:** Further investigation into the stage-specific role of glutathione synthetase can be carried out using conditional knockout systems in *Leishmania donovani.* Such insights will be crucial to understand the molecular interactions and their implications in the pathogenicity of the parasites. Expression and function of glutathione synthetase across promastigote and amastigote stages should also be evaluated using transcriptomic and proteomic approaches, especially under host-like stress conditions (e.g., oxidative burst, acidic pH).

2. ***In vivo* validation and Pharmacokinetics study:** The compounds valrubicin and ciclesonide can be evaluated on an animal model, such as BALB/c or

golden hamster infected with the wild type *Leishmania donovani* strain to access- the compounds' efficacy in reducing parasitic loads, impact on host body and synergistic effects with the current antileishmanial drugs.

3. **Development of nanoformulations of the compounds:** Nanocarrier-based delivery systems, such as liposomes or mannose-coated nanoparticles, could be engineered to enhance drug accumulation in infected macrophages, thereby improving therapeutic indices.

In conclusion, the thesis contributes significant new knowledge to the field of *Leishmania* biology and therapeutic development. By validating a novel target and identifying existing drugs with potent anti-leishmanial activity, this work not only expands our understanding of redox-based vulnerabilities in *L. donovani* but also brings us closer to developing safer, more effective, and accessible treatments for visceral leishmaniasis.