Chapter 4

Experimental Evaluation of Inhibitors Against Glutathione Synthetase and Their Impact on Parasite Survival *

Abstract

Drug repurposing has emerged as an effective strategy against infectious diseases such as visceral leishmaniasis. In the current chapter, we evaluated four out of five computationally screened FDA-approved compounds–valrubicin, ciclesonide, deflazacort, and telithromycin—for their anti-leishmanial activity on *L. donovani* parasites. The two best-performing compounds against the promastigote form of *L. donovani*, valrubicin and ciclesonide were further evaluated on the intracellular amastigote forms and tested for their inhibitory activity against recombinant *L. donovani* glutathione synthetase enzyme (*Ld*GS) expressed in a bacterial system. The findings demonstrated that valrubicin and ciclesonide have better inhibitory effects than deflazacort, telithromycin, and even the standard drug miltefosine, with notably lower IC50 values of 1.09 ± 0.09 μM and 2.09 ± 0.09 μM, respectively. This study therefore identifies ciclesonide and valrubicin as promising candidates for repurposing as anti-leishmanial agents with potential benefits over the medications that are currently available on the market. However, mechanisms of action and clinical trials of these two drugs are necessary to further investigate their safety and efficacy in treating visceral leishmaniasis.

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4.1 Introduction

Drug repurposing, also known as drug repositioning, involves the discovery of novel therapeutic applications of current or experimental pharmaceuticals. It is especially advantageous in conditions when conventional *de novo* drug development is not economically viable, or if there is an urgent necessity for a cure, such as in the pursuit of COVID-19 therapies (Pushpakom et al., 2019). Despite ranking as the second-deadliest parasitic disease following malaria, leishmaniasis is classified as among the neglected tropical diseases (NTDs) (Ghorai, 2023). Visceral leishmaniasis (VL), also known as Kala-azar, is the most fatal form of leishmaniasis. Patients often die if left untreated in 95% of the cases, as reported by WHO (World Health Organization, 2025). A significant obstacle to the efficient management of leishmaniasis is the lack of affordable, safe, and effective medicines, highlighting the urgent necessity for innovative therapeutic strategies. Despite this necessity, drug discovery for neglected tropical diseases (NTDs) encounters multiple challenges, including increased research and development (R&D) expenses, insufficient market incentives, and low success rates, which together exclude these diseases from the pharmaceutical industry (Mueller-Langer, 2013;Okwor & Uzonna, 2016b). A possible way to address these difficulties is drug repurposing, which entails discovering new uses for existing pharmaceuticals (CHARLTON et al., 2018b;Braga, 2019). This method has significant advantages, including faster development timelines, lower costs, and a reduced probability of clinical trial failure. Numerous research groups have employed drug repurposing as an approach to facilitate the discovery and development of innovative treatments for leishmaniasis by identifying new applications from existing pharmaceuticals (Freitas et al., 2023; Nath et al., 2024; Oualha et al., 2024). Approximately 30% of all new drug approvals by the US Food and Drug Administration

(FDA) over the past decade came from drug repurposing studies, and over 60% of existing drugs for leishmaniasis (Jin & Wong, 2014).

Pentavalent antimonials continue to be the principal treatment, although many repurposed medications have been utilised as secondary therapies. Amphotericin B, initially formulated as an antifungal agent, has demonstrated effectiveness against leishmanial parasites, however, it is associated with significant toxicity. The liposomal formulation has shown enhanced safety and effectiveness profiles. Pentamidine, an aromatic diamidine, is used when resistance to antimonials develops, although its therapeutic application is limited by side effects. Miltefosine, originally formulated as an anti-cancer agent, is the first orally active drug candidate approved for leishmaniasis, facilitating outpatient management; yet, concerns about toxicity, treatment failures, and the emergence of resistance remain in the endemic regions (Paliwal et al., 2021;Palić et al., 2022;Dinesh et al., 2014). High-throughput screening of drug libraries, *in silico* modelling, and system biology approaches have greatly facilitated the identification of new promising candidates with significant antileishmanial activity against different *Leishmania* species. Thus, due to the limited research and development resources given to neglected illnesses such as leishmaniasis, drug repurposing emerges as an appealing and economical strategy for developing novel therapies, potentially shortening the development timeline by 5 to 7 years (Ahmed et al., 2022; Torres-Santos, 2018; Yu et al., 2022).

The causative agents of visceral leishmaniasis are *Leishmania donovani* and *Leishmania infantum*. The parasite completes its life cycle in two stages: promastigotes, the flagellated form that resides in the lumen of a female sandfly, and intracellular amastigotes, inside the immune cells of the host (Teixeira et al., 2013). The therapeutic options for VL include chemotherapeutic drugs such as pentavalent

antimonials, pentamidine, miltefosine, amphotericin B, and its lipid formulation called AmBisome. However, these drugs are associated with several limitations, including toxic side effects, the emergence of resistance, high cost, and limited efficacy (O. P. Singh et al., 2016; Tiwari et al., 2019; Wijnant et al., 2022). Therefore, there is an urgent need to discover a novel drug target and new therapeutic approaches to combat this disease. *Leishmania* parasites can live in the toxic environment within macrophage cells by neutralizing the oxidative stress generated by the macrophages. This unique ability is contributed by the parasite's unique redox metabolism, trypanothione-based redox metabolism. In this defense mechanism, thiol-containing molecules such as glutathione and trypanothione (N1, N8-bis (glutathionyl)-spermidine) are involved (V. Ali et al., 2022; A. Kumar et al., 2019b). Trypanothione plays crucial roles in the cellular proliferation of trypanosomatids, such as the synthesis of DNA precursors, maintaining redox homeostasis, detoxification of hydroperoxides, etc. (Krauth-Siegel & Comini, 2008a). Trypanothione is biosynthesized via four ATP-dependent enzymatic steps (Figure1.4). First, L-Cys and L-Glu are conjugated by c-glutamyl cysteine synthetase (c-GCS) to generate c-glutamyl cysteine (c-GC). Subsequently, glutathione synthase (GS) ligates c-GC to Gly to generate the thiol peptide glutathione (GSH). Trypanothione synthetase (TryS) catalyzes the third and fourth steps, which involve conjugating glutathione to spermidine to produce N1-glutathionylspermidine and then adding the second glutathione molecule to N1-glutathionylspermidine to make trypanothione (Agnihotri et al., 2016). Since trypanothione-based redox metabolism is the only pathway to maintain redox homeostasis in trypanosomatids, this pathway has been well exploited for drug discovery (Battista et al., 2020; Saudagar & Dubey, 2011b). Glutathione synthetase (GS), the second enzyme in the trypanothione biosynthesis pathway, is

identified as a promising drug target in *Leishmania donovani* and other trypanosomatids due to its crucial role in the trypanothione biosynthesis pathway. Targeting *Leishmania donovani* glutathione synthetase (LdGS) gives us several advantages in therapeutic approaches. First, the low sequence similarity between the LdGS and the mammalian GS reduces the risk of host toxicity. Second, disruption of trypanothione biosynthesis and redox balance can lead to the accumulation of toxic ROS, thereby increasing the parasite's susceptibility to oxidative damage. In past studies, significant insights have been achieved in understanding the importance of GS in Trypanosoma brucei by gene-knockout methods (Pratt et al., 2014). This knowledge has opened the possibility of designing or identifying small molecular inhibitors that selectively target GS and disrupt glutathione metabolism in the parasite. In our previous study, we successfully identified FDA-approved ZINC compounds through computational methods and saw that these compounds have a strong affinity to the active site of the protein (Sarma et al., 2024). In the current study, our primary objective is to investigate the inhibitory effects of the identified compounds on the parasites. We have studied their impact on both the promastigote and amastigote forms through a series of in vitro experiments. To have a comprehensive understanding of the inhibition, we used various flow cytometric and imaging techniques. Additionally, we investigated the enzyme-inhibitory effects of the best-performing compounds using the recombinant LdGS enzyme. The enzyme was expressed in the bacterial system and the enzyme activity was performed along with inhibition studies. In summary, we aim to get valuable insights into the inhibitory potential of these compounds on the parasites and their targeted enzyme under controlled experimental conditions.

4.2 Materials and Methods

4.2.1 Materials

RPMI-1640 media (Gibco, Grand Island, NY, USA), FBS (Gibco, Diadema, Sao Paulo, Brazil) and streptomycin–penicillin antibiotic solutions (Gibco), M199 media, Thiazolyl Blue Tetrazolium Bromide, DMSO, Miltefosine, Phorbol 12-myristate 13-acetate (PMA), Poly-Llysine coated coverslips, γ-glutamyl cysteine, Phosphoenol pyruvate (PEP) and Lactate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO, USA) drug compounds (Med Chem Express, Monmouth Junction, NJ, USA) restriction enzymes (NEB, Ipswich, MA, USA), ligase enzyme (Takara, Kusatsu city, Japan). Other chemicals used in the study were commercially available.

4.2.2 Parasites and Cell Line

Leishmania donovani promastigote cells (MHOM/IN/1983/AG83) were cultured at 25 °C in M199 media (Ph 7.4) supplemented with 10% FBS (heat-inactivated), 0.35 g·mL1 $NaHCO_3$, 22 mM HEPES Buffer, and 1% Penicillin–Streptomycin antibiotic. For each experiment, *L. donovani* promastigote cells were cultured to the logarithmic phase and 2 $x10^6$ /mL cells were for the study. The J774A.1cell line (murine macrophage) and the THP-1 cell line (human monocytes) were maintained at 37 °C and 5% CO2 in RPMI-1640 media (pH 7.4), supplemented with 10% FBS, 1% Penicillin–Streptomycin, and Gentamycin antibiotic. THP-1 monocyte cells were induced to differentiate into adherent macrophages by treating them with Phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) overnight at 37 °C in a 5% CO2 incubator. For cloning and expression purposes of recombinant LdGS protein, pET28a vector and *E. coli* competent cells of strain DH5a, and BL21(DE3) were used.

4.2.3 Anti-Leishmanial Activity

Leishmania donovani promastigote cells were incubated with gradients (ranging from 0.09 to 100 μM) of the four FDA-approved drug compounds- valrubicin, ciclesonide, telithromycin, and deflazacort. These four drugs have been selected based on the results obtained in our previous study (Sarma et al., 2024). For negative control, cells were incubated only in M199 media without drugs. After 48 h of incubation, the MTT assay was performed as described in (Saudagar et al., 2013) and the IC50 values of each compound were determined using GraphPad Prism version 5.0 (trial version) dose–response curve. To check the efficacy of the two promising drugs in eliminating intracellular amastigotes, the anti-amastigote assay was performed using THP-1-derived macrophage cells. Briefly, THP-1 monocyte cells at a concentration of 2 9 105 cells·mL1 were seeded in a 12-well plate containing RPMI 1640 media in a 5% CO2 incubator. Monocytes were differentiated into adherent macrophages using 100 ng·mL1 of PMA for 12 h (Saha et al., 2020). Subsequently, parasites were added at a 1:10 ratio of macrophage to parasites and incubated for 12 h. After 12 h, unbound parasites were removed by washing with 19 PBS, and the macrophages were incubated with a gradient of valrubicin and ciclesonide (ranging from 0.09 to 100 lM) for 48 h. After 48 h, cells were fixed with chilled methanol for 1 h, followed by Giemsa staining. Intracellular amastigotes were then counted in 100 randomly selected macrophages for each well. The resultant data were plotted to calculate the IC50 values of each compound using GraphPad Prism version 5.0 (trial version) dose-response curve (Ilaghi et al., 2021).

4.2.4 Cloning and Expression of Recombinant *LdGS* Gene

The nucleotide sequence of the glutathione synthetase gene of *Leishmania donovani* (accession number XM_003859416) was obtained from the NCBI nucleotide database and amplified by PCR using the designed forward (5′-ATGAATTCATGCCCGCCACGACGACG-3′) and reverse (5′-ATCTCGAGGATTATCTGCACGCTGTC-3′) primers from the genomic DNA of *L. donovani*. The resultant gene fragment was double-digested by EcoRI and XhoI restriction enzymes and then ligated into the pET28a vector. Subsequently, the constructed plasmid was introduced into DH5α *E. coli* cells to achieve stable transformation. Positive clones were screened by PCR and restriction digestion techniques. Final confirmation was achieved by Sanger sequencing before the transformation of the plasmid into the *E. coli* BL21 (DE3) competent cells for expression. For expression, cells were inoculated and grown at 37 °C until they reached an OD of 0.6 at 600 nm. Subsequently, the protein was overexpressed by 0.1 mM IPTG induction for 16 h at 18 °C. After harvesting by centrifugation at 9300 g for 3 min at 4 °C, cells were resuspended in a lysis buffer (50 mM Tris, 200 mM NaCl, 2 mM b-mercaptoethanol, 1 mM PMSF, and 0.5 mg·mL1 lysozyme) and incubated for 30 min at 4 °C. Subsequently, cells were lysed through sonication for 40 min (5 s on and 7 s off at 45% amplitude) in an ice bath. The resulting cell debris was separated by centrifugation at 2300 g for 10 min. The crude protein contained in the supernatant was estimated through the Bradford method (Brady & Macnaughtan, 2015) using BSA as a standard and run on 10% SDS gel for visualization.

4.2.5 Relative Activity and Inhibition Studies of LdGS

The soluble fractions of the expressed LdGS were collected for the steady-state kinetic analysis and inhibition study. The LdGS activity study was performed using the ATP-coupled assay, as described in previous literature (Dinescu et al., 2010; Pratt et al., 2014). All assays were performed at 25 °C in 96-well plates using a microtiter plate reader (BioTek Synergy HT). For each assay, 100 mM Tris–HCl (pH 8.2), 20 mM MgCl2, 50 mM KCl, 250 μM NADH, 5 mM phosphoenolpyruvate, 10 U of lactate dehydrogenase, and 10 U of pyruvate kinase were all included in the 200 μL total reaction volume. The following substrate concentrations were used to measure LdGS activity in steady-state kinetics: 3 mM glycine, 0.2 mM c-glutamyl cysteine, and 0.2 mM ATP Reactions were monitored at 340 nm. To study the inhibitory effect of selected compounds on LdGS activity, the LdGS concentration was maintained at 0.5 mg/mL1. The compounds were dissolved in DMSO and incubated with LdGS for 30 min. Inhibitor concentrations ranging from 0.5 to 50 μM were used, with DMSO used as the control. The relative activity (%) in the presence or absence of an inhibitor was determined. The IC50 values of the selected inhibitors were determined by plotting the inhibitor concentration on the X-axis and relative activity (%) on the Y-axis, followed by fitting the data to non-linear regression analysis using GraphPad Prism5 (trial version).

4.2.6 Cytotoxicity Assay

The cytotoxicity of the best FDA-approved molecules (valrubicin and ciclesonide) was tested with the mouse-macrophage cell line, J774a.1 by the MTT assay. For that, 2 9 105 cells·mL1 of macrophage cells were incubated in RPMI 1640 media with a gradient concentration of the drug compounds in a 5% CO2 incubator. After 48 h of

incubation, the MTT assay was performed using the procedure previously discussed, and the CC50 values were determined by plotting GraphPad Prism version 5.0's dose–response curve (Saudagar & Dubey, 2014b).

4.3 Results

In our previous study, we reported a few FDA-approved ZINC compounds through computational methods (Sarma et al., 2024). The current manuscript reports experimental validation and insight into molecular mechanisms leading to parasitic death by these selected compounds.

4.3.1 Compounds Inhibit *L. donovani* Promastigotes at Varying Concentrations

In the viability assay of *L. donovani* promastigotes, it was observed that the compounds valrubicin and ciclesonide inhibited the parasites' viability at very low concentrations, approximately 90% of the cells died at 1.5 µM and 3.5 µM, respectively. In contrast, deflazacort and telithromycin were seen to display weak inhibitory effects, requiring higher concentrations of approximately 75.67 µM and 152.39 µM, respectively, to kill 90% of the cell population. To further illustrate their inhibitory potency, IC50 values for each compound were calculated from the dose–response curve. Valrubicin and ciclesonide exhibited lower IC50 values of 1.09 ±0.09 µM and 2.09 ±0.09 µM, respectively, compared to deflazacort and telithromycin with IC50 values of 35.66 ±0.73 µM and 108.65 ±4.41 µM, respectively (Figure 4.1). These findings demonstrate the superior anti-promastigote activity of valrubicin and ciclesonide, establishing them as promising candidates for further study.

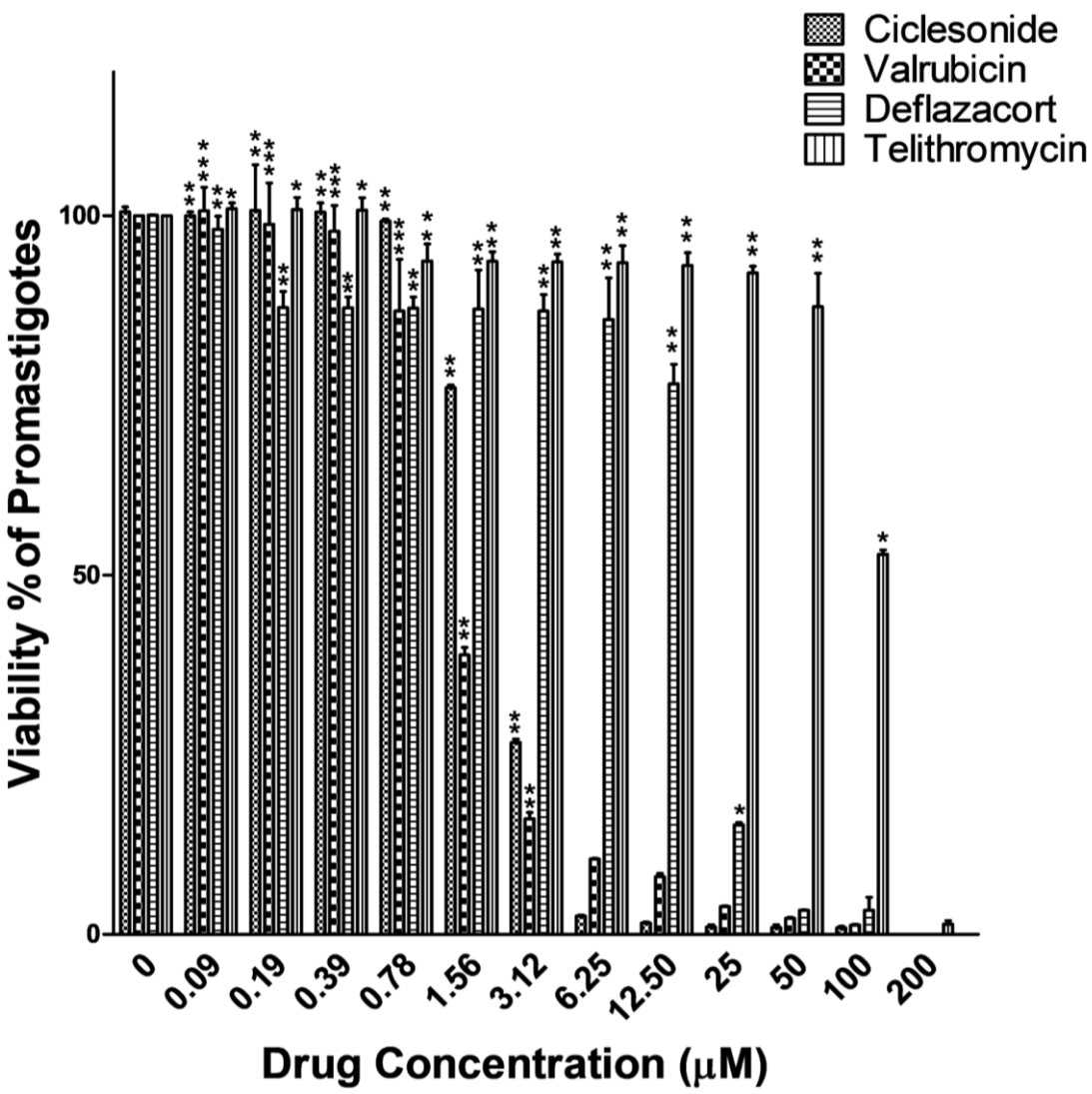

Figure 4.1: Anti-leishmanial activity of selected compounds on *Leishmania donovani* promastigotes. Four selected FDA-approved compounds were tested (concentrations 0.09–200 μM) on promastigote forms of *Leishmania donovani* for 48 h. The untreated sample (0 μM) was used as a negative control for each drug. The bar graph represents the mean ± SEM of the data from three independent experiments. For all the experiments, the significance level in comparison to the control was determined by the one-way ANOVA test using GraphPad Prism5 (trial version), with *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001 denoting the levels of significance.

The anti-leishmanial property of valrubicin and ciclesonide, best compounds on promastigotes, was tested against intracellular amastigotes through Giemsa-stained macrophages observed under a light microscope. When exposed to the macrophages, flagellated promastigotes transformed into the nonmotile amastigotes within

parasitophorous vacuoles of macrophages. This parasitic load into the macrophage cells serves as a pathogenesis index. To evaluate the drug efficacy, it is important to examine its impact on macrophage-resident amastigotes (Islamuddin et al., 2022). Treatment with varying concentrations of valrubicin and ciclesonide resulted in a dose-dependent reduction in amastigote load within macrophages, with IC50 values of 1.74 ±0.05 μM and 3.32 ± 0.21 μM, respectively (Figure 4.2). Giemsa-stained samples of infected and treated macrophages revealed nearly complete clearance of intracellular amastigotes at higher doses under a light microscope. This demonstrates the potential of valrubicin and ciclesonide in effectively combating intracellular amastigotes and supports their consideration as promising candidates for anti-leishmanial therapeutics.

4.3.2 Valrubicin and Ciclesonide Exhibit Moderate Cytotoxicity to Host Cells

Valrubicin and ciclesonide are FDA-approved drugs and their safety is already been evaluated in the literature. However, after observing the promising inhibitory impact on the pathogen, ensuring the pharmaceutical safety of any drug is essential (Bácskay et al., 2018). For that, the cytotoxic evaluation of valrubicin and ciclesonide was carried out on J774A.1 mouse macrophage cell line. CC50 values of valrubicin and ciclesonide were found to be 4.71 ± 0.08 μM and 15.76 ± 0.09 μM, respectively (Figure 4.3). The selectivity index for each drug was calculated from the CC50/IC50 ratio for both promastigotes and amastigotes (Table 4.1). Selectivity index quantifies the margin between efficacy and toxicity, directly guiding the selection and advancement of new therapeutic agents. Drugs with higher SI values are more likely to be safe and effective, underscoring the SI's critical role in drug development and therapeutic application. SI > 10 is generally considered a strong indicator of specificity and safety.

In such a case, the compound is much more toxic to target cells than to normal cells, making it suitable for drug development. While SI 2–10 generally suggests moderate selectivity, compounds may have therapeutic potential but require careful evaluation. For SI < 2, indicates low selectivity; the compound's effect on normal cells is too close to its effect on target cells, raising toxicity concerns. Notably, ciclesonide shows a higher selectivity index compared to valrubicin, indicating it a safer drug candidate for the host than valrubicin.

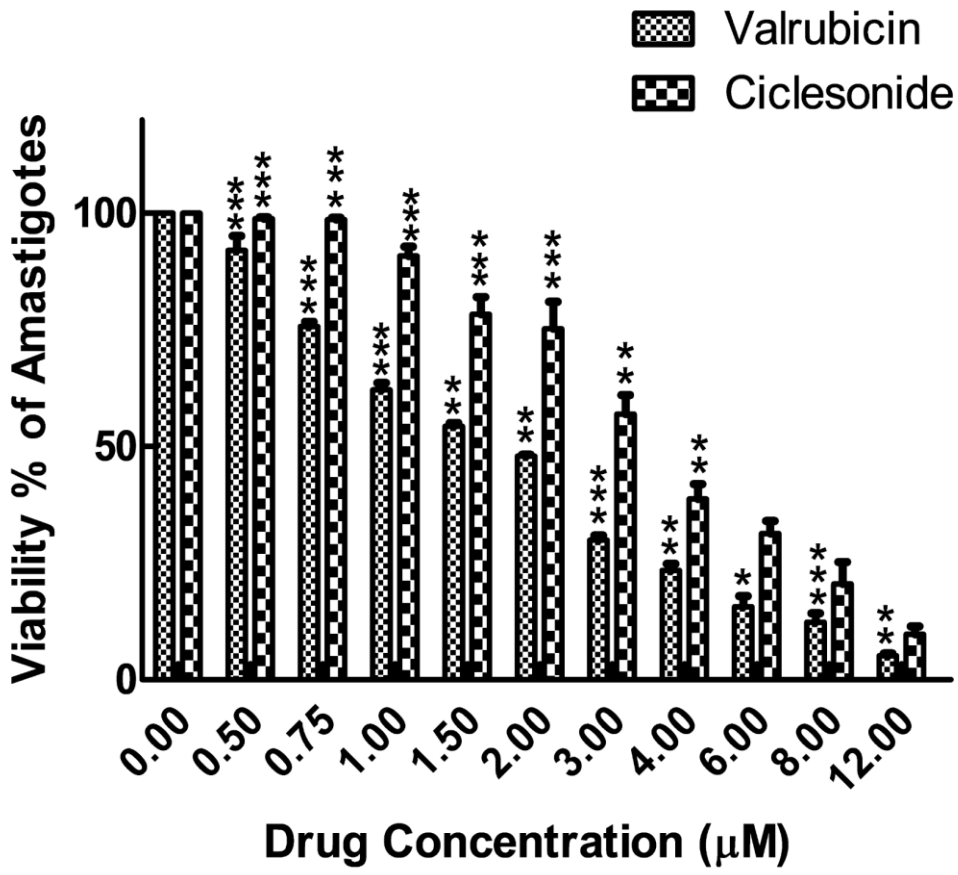

Figure 4.2: Anti-leishmanial activities of valrubicin and ciclesonide were evaluated on the *Leishmania donovani* amastigotes. Macrophages were differentiated from THP-1 cell line, infected with *Leishmania donovani* promastigotes, and the amastigotes residing inside the macrophages were treated with compound valrubicin and ciclesonide with concentrations ranging from 0.5 to 12.0 IM for 48 h. Intracellular amastigotes were fixed, stained with Giemsa, and counted under the light microscope to find the parasitic load. The bar graph represents the mean ± SEM of the data from three independent experiments. For all the experiments, the significance level in comparison to the control was determined by the one-way ANOVA test using GraphPad Prism5 (trial version), with *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001 denoting the levels of significance.

Table 4.1: Selectivity index of FDA-approved drugs, valrubicin and ciclesonide, on J774A.1

Drug compound	IC50 for promastigotes (μM)	IC50 for amastigotes (μM)	Cytotoxicity (CC50) for macrophages (μM)	Selectivity index for promastigotes (CC50/IC50)	Selectivity index for Amastigotess (CC50/IC50)
Valrubicin	1.09 ± 0.09	1.74 ± 0.05	4.71 ± 0.08	4.32 ± 0.55	2.71 ± 1.6
Ciclesonide	2.09 ± 0.09	3.32 ± 0.21	15.76 ± 0.09	7.54 ± 1.0	4.75 ± 0.43

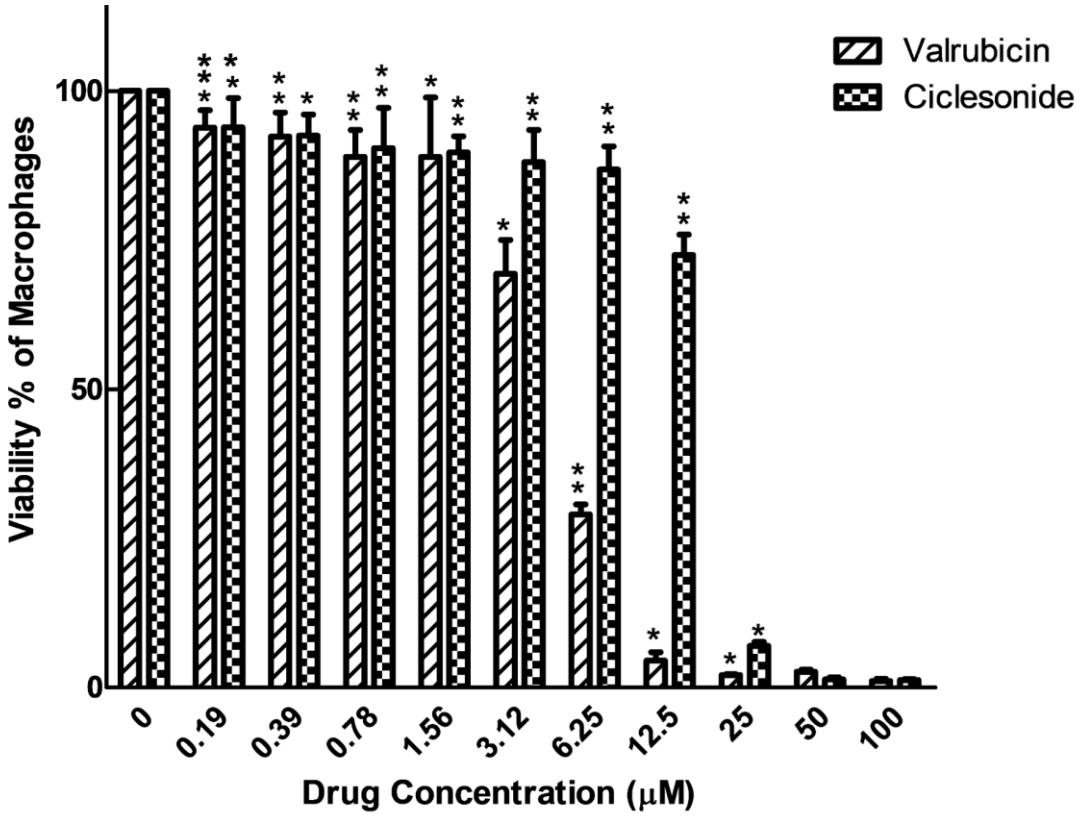

Figure 4.3: Cytotoxicity test of FDA-approved drugs, valrubicin and ciclesonide, on the J774A.1 murine macrophage cells. Murine macrophage cell lines were treated with a dose range of 0–100 μM for 48 h. The untreated sample (0 μM) was used as a control. The bar graph represents the mean ± SEM of the data from three independent experiments. For all the experiments, the significance level in comparison to the control was determined by the one-way ANOVA test using GraphPad Prism5 (trial version), with *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001 denoting the levels of significance.

4.3.3 Cloning and Expression of Recombinant *L. Donovani* Glutathione Synthetase (*Ld*GS)

The 1821 bp coding sequence of the *Ld*GS gene was successfully cloned into the PET28a vector and was confirmed through restriction digestion using EcoRI and XohI. Further validation of the LdGS cloning was done through Sanger sequencing. The overexpression of the LdGS protein was carried out in BL21 (DE3) *E. coli* cells as described in the methodology section. The crude LdGS protein was 67.1KDa in size and was verified from the SDS gel [Figure 4.4 (B)]. The protein concentration was found to be ~20 mg·L1 of the bacterial culture.

4.3.4 Activity of the Recombinant LdGS Enzyme was Inhibited by Valrubicin and Ciclesonide

The expressed LdGS in the cell lysate after the removal of cellular debris and non-lysed cells was collected. The cell lysate was subsequently used for the determination of LdGS activity by ATP-coupled assay as described in the past literature (Dinescu et al., 2010). Notably, a linear correlation was observed between enzyme concentration and its activity, reaching a maximal value at $1\mathrm{mg}/\mathrm{ml}$.

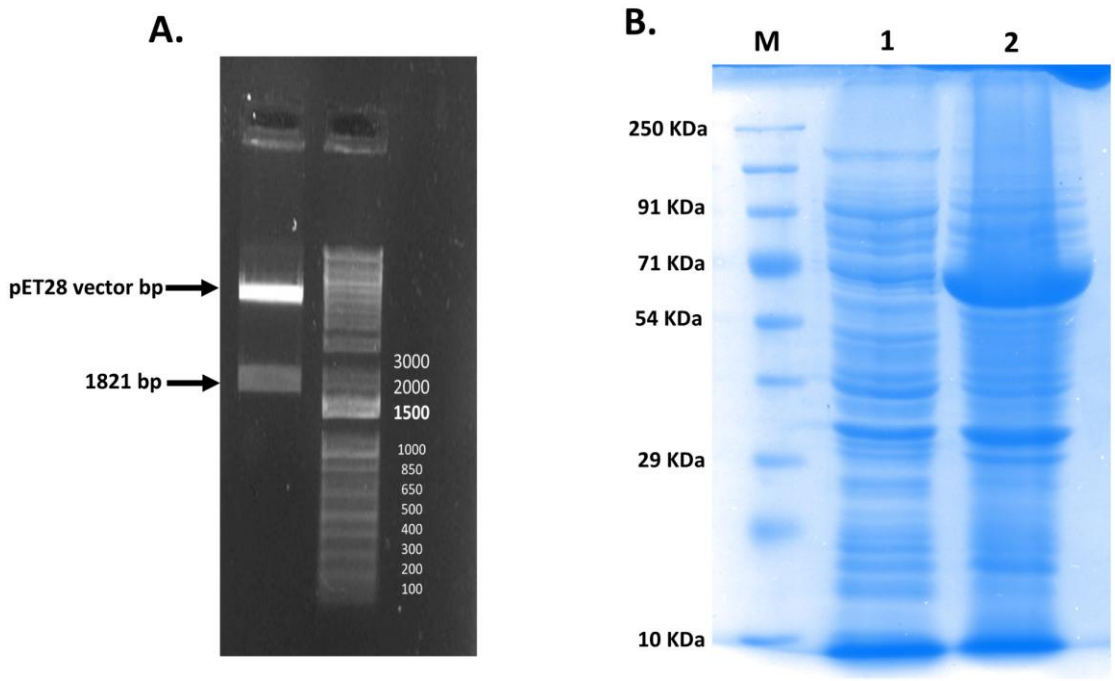

Figure 4.4: Cloning and expression of recombinant *Leishmania donovani* glutathione synthetase in *E. coli.* (A) Confirmation of the cloning of the *Leishmania donovani glutathione synthetase* gene into the pET28a (+) vector by restriction digestion, releasing the 1.82kb insert. (B) Expression analysis of the recombinant glutathione synthetase gene in *E. coli* BL21 (DE3) cells. Lane M: protein molecular weight marker; lane 1: uninduced cell lysate; lane 2: IPTG induced cell lysate.

Upon examination of the soluble fractions containing LdGS at a concentration of 0.5 mg/mL, it was observed that the relative activity was approximately ~40% in comparison to the reference condition. This observation suggests a concentration-dependent modulation of LdGS activity in the soluble fractions and an indicative of regulatory mechanisms or saturation effects within the enzyme-substrate system. The relative inhibitory effect of the selected inhibitors on LdGS was assessed at fixed concentrations of LdGS (0.5 mg/mL). The half-maximal inhibitory concentrations (IC50) were derived from the dose–response curve [Figure 4.5 (B) and (C)]. The IC50 values for valrubicin were determined to be 16.11 µM, while those for Cislesonide were established at 10.56 µM against the target protein *Ld*GS.

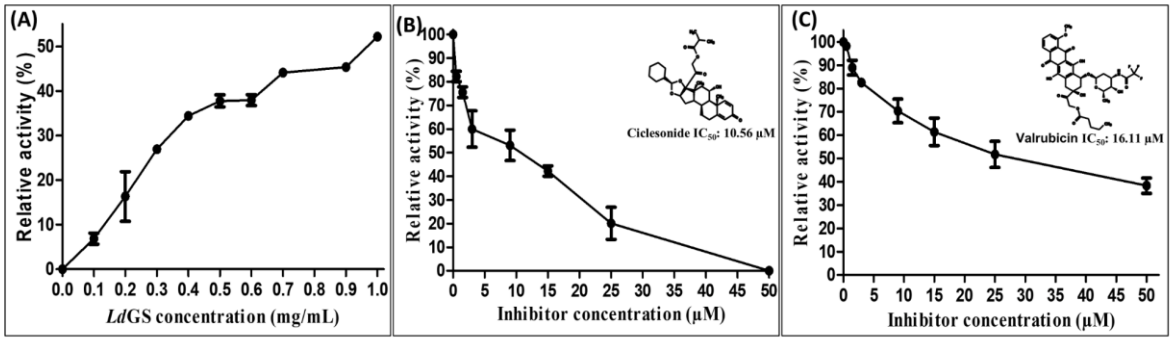

Figure 4.5: The enzymatic activity and Inhibition of *Leishmania donovani* glutathione synthetase (LdGS). Overexpressed recombinant Ld GS cloned in pET 28a vector and transferred in *E. coli* BL21 cells (DE3) was used in the study (A) illustrates that LdGS soluble fractions exhibit a linear correlation between activity and LdGS concentration within the range of 0.1 to 1 mg/ml. (B) ciclesonide (C) valrubicin, demonstrate enzyme activity in the presence of varying concentrations of Ciclesonide and Valrubicin, ranging from 0.5 to 50 µM. All assays were performed in triplicate and the IC50 value for each compound was calculated through dose–response curves generated using GraphPad Prism5 (trial version). Error bars in each of the three graphs represent the standard error of the mean (SEM).

4.4 Discussion

Infectious diseases stand as the third major cause of death globally. Leishmaniasis, the second most neglected tropical disease after malaria, poses a significant public health concern. In the Indian subcontinent, Visceral leishmaniasis or Kala-azar caused by *Leishmania donovani* is the prominent form. Due to the unavailability of vaccines (Dinc, 2022), the current treatment options are limited to chemotherapeutic drugs which have many limitations including drug resistance, creating an urgent need to identify a new drug that can effectively eradicate the disease. It is expected that the drug against a new target likely to work better against drug resistance strains.

Drug repurposing is an effective strategy for infectious diseases like visceral leishmaniasis reducing the cost and time for drug development (Hamid et al., 2024). Examples of repurposed drugs for leishmaniasis include Miltefosine, Amphotericin B,

and Pentamidine, all of which were originally designed or approved for other medical purposes (Jones et al., 2018). In the rational drug discovery process, the most important step is to identify a drug target or pathway that is very crucial to the pathogen's survival. *Leishmania* parasites enter the host's macrophage cells, where they multiply and flourish even in the acidic environment within these cells. However, it was not clear exactly how the parasite manages to survive and even evade different reactive oxygen species (ROS) in the parasitophorous vacuole of these cells. Recent studies discovered that trypanothione-based redox metabolism plays a crucial role in fighting against ROS produced (Colotti et al., 2020; Raj et al., 2020). One of the important enzymes of this pathway is glutathione synthetase, which belongs to the ATP-grasp superfamily and plays a key role in the biosynthesis of glutathione (GSH), the precursor of the main metabolite, trypanothione (Walker & van der Donk, 2016). The structural differences of human and parasitic glutathione synthetase allow us to target the enzyme as a suitable drug target. In our previous study, the computational analysis revealed that five FDA-approved Zinc candidates; valrubicin, ciclesonide, deflazacort, Simeprevir, and telithromycin show good binding affinities to LdGS (Sarma et al., 2024). The present study is the continuation of the previous study and explains an in vitro assay on *Leishmania donovani*, to see the efficacy of four selected drugs. It is important to note that one of the compounds, Simeprevir was already screened on *Leishmania donovani* and was found to target sterol C-24 methyltransferase (Tabrez et al., 2021). In the current study, from the MTT assay on promastigotes, two out of four drugs- valrubicin and ciclesonide had very low IC50 values of 1.09 ± 0.09 µM and 2.09 ± 0.09 µM, respectively, while the drug deflazacort and telithromycin exhibited IC50 values of 35.66 ±0.73 µM and 108.65± 4.41 µM, respectively. The IC50 values of valrubicin and ciclesonide are notably lower than that of the standard drug Miltefosine,

which is approximately 13.6 μM (Ranjan & Dubey, 2023), establishing them as good candidates for further study. Valrubicin, derived from doxorubicin as an Ntrifluoroacetyl 14-valerate derivative, is renowned for its anti-tumor properties, acting by disrupting DNA repair through histone loss in tumor cells (Onrust & Lamb, 1999b). In contrast, ciclesonide is utilized for treating inflammatory conditions like asthma and allergic rhinitis. This nonhalogenated glucocorticoid undergoes conversion by intracellular esterases into desisobutyryl-ciclesonide, which binds to cytoplasmic glucocorticoid receptors, thereby modulating expression of anti-inflammatory genes and inhibiting pro-inflammatory cytokines (Joerger et al., 2009; Maruta & He, 2020).

Upon observing significant parasitic death at lower concentrations of valrubicin and ciclesonide, we examined their inhibitory effects on crude recombinant glutathione synthetase enzyme. Although the recombinant enzyme expressed in bacterial systems was not obtained in purified form, these drugs were qualitatively assessed using crude lysate from over-expressed bacteria. Both compounds demonstrated inhibitory effects on enzyme activity in the crude sample, corroborating their target-based inhibition. Consequently, to assess the efficacy of valrubicin and ciclesonide against intracellular amastigotes and elucidate their mechanisms of action against promastigotes, further in-vitro assays were conducted.

The infectious metacyclic promastigotes enter into the host's macrophages, initiating the formation of *Leishmania* parasitophorous vacuoles (LPVs). Within these LPVs, the parasites undergo differentiation, transforming into non-motile amastigote forms (Young & Kima, 2019). We tested the candidates, valrubicin and ciclesonide for anti-amastigote activity using TPH-1 differentiated Macrophage cells and found that both the candidates can effectively inhibit the intracellular amastigotes at very low concentrations, with IC50 values 1.74 ± 0.05 μM and 3.32 ± 0.21 μM, respectively.

Additionally, cytotoxic effects on the host cells were studied for these two drugs using J774A.1 murine macrophage cells. Based on the selectivity index, it was found that valrubicin exerts a comparatively higher cytotoxic effect as compared to ciclesonide. After promising indications of the two drugs in parasitic inhibition, the enzyme inhibition study was conducted using the recombinant LdGS enzyme (Figure 4.5). Inhibition was observed for both drugs, where ciclesonide exhibited an IC50 value of 10 μM, while valrubicin had a slightly higher IC50 value of 16 μM, indicating higher side effects to host cells. Nevertheless, through this study, we have identified two new compounds that can selectively inhibit the parasite's glutathione synthetase enzyme, which can ultimately cause the parasite's death at very low concentrations. The identified compounds are designed to disrupt the parasite's redox equilibrium, thus exposing it to oxidative stress. However, to investigate the mechanism of action of these compounds, cell-based biochemical and imaging assays can be carried out.