Chapter 5

Mechanism of Cell Death and Biochemical Alterations in *Leishmania donovani* *

Abstract

Assessing the therapeutic potential of new antileishmanial drugs depends on understanding the mechanisms of cell death induced by the identified compounds. Antileishmanial drugs can exert their effect in diverse cell death phenotypes, including apoptosis-like programmed cell death, autophagy, and necrosis, depending upon the drug dose and developmental stage of the parasite. Examining these processes is crucial for determining drug specificity, minimizing host toxicity, and anticipating resistance development. In this chapter, we provide a comprehensive mechanistic analysis of two potential compounds, i.e.-valrubicin and ciclesonide, identified by *in vitro* screening. Both these compounds significantly induced intracellular reactive oxygen species (ROS) in *Leishmania donovani* promastigotes, resulting in oxidative stress, DNA fragmentation (demonstrated by DNA laddering), cell cycle arrest, and characteristics indicative of apoptosis-like cell death. Elucidating these pathways offers critical insights into the parasite biology and pharmacological mechanisms, facilitating the rational development of more potent therapies. This study shows potential for creating new antileishmanial therapies with improved effectiveness and less host toxicity.

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5.1 Introduction

Leishmaniasis has prompted the formulation of various medications, including sodium stibogluconate, miltefosine, pentamidine, and Amphotericin B, with further drug-repurposing trials currently in progress. An efficient anti-leishmanial is one that targets the parasite specifically and can successfully eradicate it. Due to the resemblance in metabolic pathways between *Leishmania* and its host, a drug that specifically targets the parasite without affecting the host is not always feasible. However, because *Leishmania* proliferates more rapidly than the host, such drugs can effectively eradicate the parasite at non-cytotoxic doses for the host. These pharmaceuticals, owing to their reduced dosage and extended exposure duration, elicit morphological and biochemical characteristics indicative of controlled cell death in the parasites. The emergence of the TriTryp genome has led to the identification of numerous effector molecules and their associated pathways; nevertheless, thorough understanding of these pathways remains insufficient. Numerous newly discovered small compounds and inhibitors can efficiently eradicate parasites by activating controlled cell death processes in a dose- and time-dependent manner. The mechanism of drug-induced cell death pathways is contingent upon the nature of the stimuli, the drug's method of action, the parasite subspecies, and its developmental stage. The unregulated and uncontrolled use of medications has resulted in the development of chemoresistance by circumventing drug-induced stress (Ponte-Sucre et al., 2017). Understanding the progressive emergence of chemoresistance by circumventing cellular apoptosis pathways is crucial to avert such resistance. Comprehending all discrete mechanisms of cell death and their associated properties is crucial for investigating resistance development and formulating an appropriate treatment solution.

5.2 Materials and Methods

5.2.1 Materials

RPMI-1640 media (Gibco, Grand Island, NY, USA), FBS (Gibco, Diadema, Sao Paulo, Brazil) and streptomycin–streptomycin-penicillin antibiotic solutions (Gibco), M199 media, Thiazolyl Blue Tetrazolium Bromide, DMSO, Miltefosine, Phorbol 12-myristate 13-acetate (PMA), H2DCFDA (Sigma), N-acetyl-L-cysteine, Propidium iodide (Thermofisher), Annexin-V/FITC apoptosis kit, RNAase (HiMedia)

5.2.2 Scanning Electron Microscopy (SEM) Analysis

To observe the parasite's morphological changes after drug treatment, SEM imaging was used (R. Ali et al., 2021; V. Kumar et al., 2021). Briefly, 2 x105 cells·mL1 of *L. donovani* promastigote cells were incubated with IC50 and 2xIC50 concentrations of valrubicin and ciclesonide for 48 h. Subsequently, cells were harvested by centrifugation and washed twice with ice-cold 1XPBS. Cells were then fixed with fixative solution (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer) for 1 h, poured on poly L-lysine coated coverslips, and incubated for 6 h at 25 °C. To remove the unadhered cells and any buffer salts, the coverslips were washed with sterile filtered water. The coverslips were then dehydrated with an ascending series of ethanol (30%, 50%, 70%, and 90%) and air-dried for 30 min. Coverslips were then mounted on a metallic stump by carbon nanotube and coated with gold–palladium. The SEM image was taken by using Carl Zeiss EVO-18.

5.2.3 Reactive Oxygen Species Analysis

The intracellular reactive oxygen species (ROS) generated upon drug treatment were assessed through a flow cytometer using H2DCFDA fluorescent dye. Briefly, *L. donovani* promastigote cells ($2x10^5$ cells/mL) were incubated with 50 µM of H2O2 (positive control), 10 µM of N-acetyl-L-cysteine (negative control), IC50 and 2x IC50 concentrations of valrubicin and ciclesonide for 48 h. Post incubation, cells were harvested and washed twice with ice-cold 19 PBS followed by incubation with 10 µM of H2DCFDA in 500 µL of 19 PBS for 20 min in a dark environment at room temperature. Subsequently, fluorescent intensity resulting from ROS-mediated fluorescence was quantified through the Beckman flow cytometer at an excitation/ emission wavelength of 488/522 nm. For each sample, a total of 10 000 events were recorded.

5.2.4 Flow Cytometric Analysis of Cell Cycle

To study the effect of drugs on the progression of the cycle in promastigote cells, flow cytometric analysis was carried out by using Propidium Iodide (PI) dye [22,24]. For that, promastigote cells were treated with IC50 and 2x IC50 concentrations of ciclesonide and valrubicin as mentioned above. After treatment, cells were collected, washed twice with 19 PBS, and fixed with 70% ethanol for overnight at 4 °C. Fixed cells were washed with 19 PBS and incubated with 500 lL of RNase A (200 lg·mL1) at 37°C for 1 h. After the incubation with RNase A, Propidium iodide was added to the solution at a concentration of 50 lg·mL1 and again incubated in a dark room for 20 min, subsequently, cells were subjected to the flow cytometer (Beckman, Indianapolis, IN, USA). A total of 10 000 events were recorded to generate histograms.

5.2.5 DNA Laddering Assay

Post-drug treatment DNA laddering analysis often provides insights into the therapeutic impact on cellular events in *Leishmania* parasites. We performed the DNA laddering assay on *L. donovani* cells, subjecting them to incubation with H2O2 (as a positive control), IC50, and 2x IC50 concentrations of valrubicin and ciclesonide. Untreated cells were considered as a negative control. Following 48 h of incubation, cells were harvested, suspended in a lysis buffer, and subjected to DNA extraction by the Phenyl-chloroform method. The extracted DNA was electrophoresed on a 1.5% agarose gel at 60 V for 1 h.

5.2.6 Annexin V/PI dual staining for apoptosis/ necrotic study

To confirm the nature of cell death of the promastigotes, the apoptosis analysis using Annexin-V/PI dual staining was conducted. Promastigote cells were treated with IC50 and 2x IC50 doses of valrubicin and ciclesonide for 48 h. Miltefosine, a prescribed drug for visceral leishmaniasis, was used as a positive control at its IC50 and 2x IC50 doses, and its capacity to induce apoptosis in *Leishmania* has been established. Untreated cells were considered as a negative control. Post-treatment, cells were washed twice with 19 PBS, stained with Annexin V and PI according to the manufacturer's protocol, and analyzed using the flow cytometer. FITC-A (Ex/Em – 488 nm/525 nm) and PE-A (Ex/Em – 488 nm/575 nm) were used for the detection of apoptotic cells and necrotic cells, respectively. A total of 10,000 events were recorded for each sample to generate adequate data for the study.

5.3 Results

5.3.1 Drugs Alter the Cellular Morphology of *L. donovani* Promastigotes The morphological alteration after the drug treatment is an important indication of the parasites' response to the drug, revealing cell death mechanisms, such as apoptosis or mitotic catastrophe (Chhajer et al., 2024; Neto et al., 2011). The Scanning Electron Micrographs of the *L. donovani* promastigotes post drug treatment, demonstrated that while untreated parasites typically have an elongated flagella and a slender body shape, exposure to an IC_{50} dose of ciclesonide causes the parasite's body length and flagella to be significantly reduced. Parasites treated with an IC_{50} dose of valrubicin had more intense cellular damage, where no proper body structure and flagella were observed (Figure 5.1).

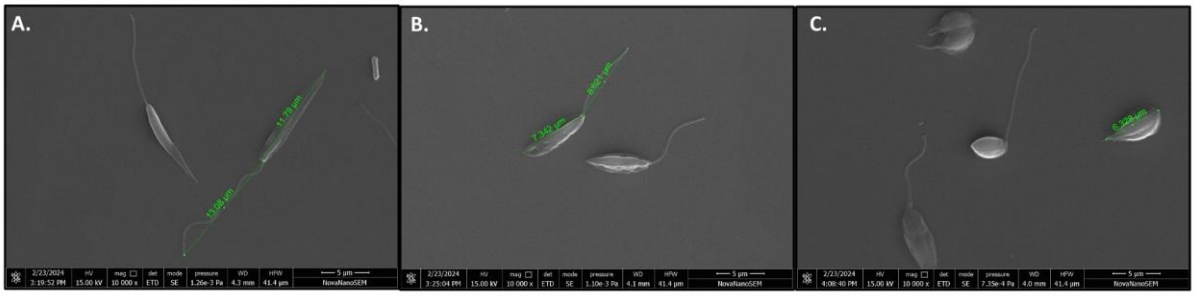


Figure 5.1: Effect of valrubicin and ciclesonide on morphological changes of *L. donovani* promastigotes observed under Scanning Electron Microscope (SEM). (A) The SEM micrograph of untreated promastigotes with elongated and slender body shapes with healthy flagellum. (B) SEM micrograph of promastigotes treated with ciclesonide at its IC50 concentration shows altered morphology and significantly shorter flagellum. (C) SEM image of promastigotes treated with valrubicin at its IC50 concentration showing much shorter flagellum and deformed morphology.

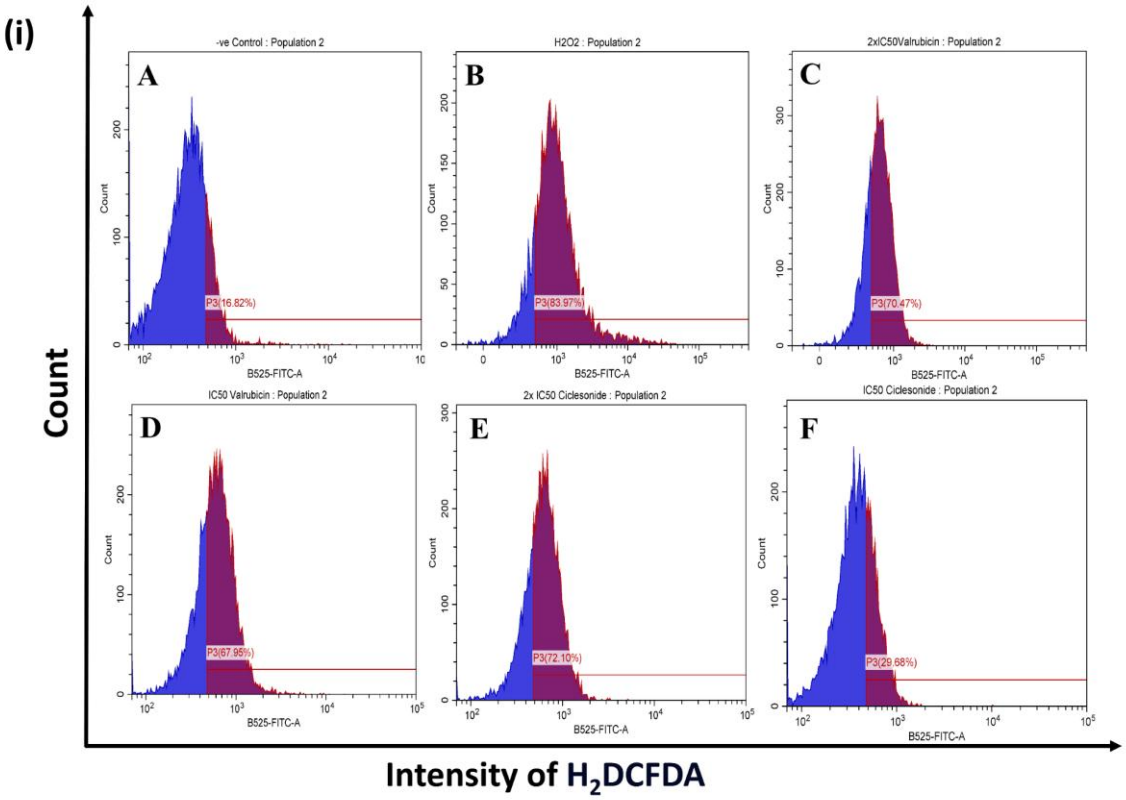
5.3.2 Intracellular ROS of *L. donovani* Promastigotes in Dose-dependent Manners

The assessment of intracellular ROS generation within the parasitic cells after drug exposure is a valuable indicator of cellular stress. Since the drugs under investigation specifically target the glutathione synthetase enzyme, a key component in the trypanothione synthetase pathway known to reduce oxidative stress in parasites, it is hypothesized that intracellular ROS levels would increase after drug exposure. Using H_2DCFDA staining for ROS detection, N-acetylcysteine (NAC)- treated cells (-ve) exhibited approximately 15.64% ROS positivity, whereas H2O2-treated cells (+ve control) displayed a higher ROS positivity, reaching nearly 84.71%. Notably, valrubicin-treated cells displayed a dose-dependent response, with 77.82% and 63.75% ROS positivity observed for 2xIC_{50} and IC_{50} doses, respectively (Figure 5.2). Similarly, ciclesonide-treated cells exhibited a dose-dependent pattern, with 70.67% and 37.71% ROS-positive cells for 2xIC50 and IC50 doses, respectively. These findings clearly demonstrate a dose-dependent increase in the ROS generation, supporting the hypothesis that upon inhibition of the glutathione synthetase by these two drugs disrupts the parasite's redox equilibrium that leads to oxidative stress (Afrin et al., 2019).

5.3.3 Drugs Arrest the Cell Cycle of the Parasite at G_0/G_1 Phase

Studying the dynamics of the cell cycle progression in drug-treated *Leishmania donovani* promastigotes reveals crucial insights into the mechanism of drug action. In our study, we observed significant effects of both drugs, ciclesonide and valrubicin, on the progression of the cell cycle when compared to untreated control cells. The percentage of cells in the subG1 and G0/G1 was increased in a dose-dependent

fashion for both drug-treated cells. In the untreated control cells, approximately 29.31% of cells were in G0/G1 phase [Figure 5.3 I(A)]. In ciclesonide-treated cells, approximately 37.82% and 49.08% of cells were in G0/G1 phase for IC50 and 2x IC50 doses, respectively [Figure 5.3 I(B) and 5.3



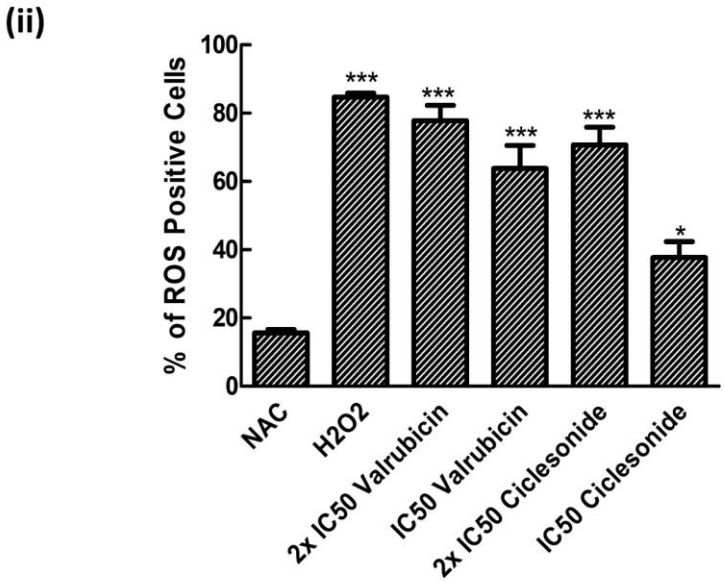


Figure 5.2: Flow cytometric assessment of ROS generation in drug-treated *L. donovani* promastigotes. Parasites were incubated with 2xIC50 and IC50 concentrations of valrubicin and ciclesonide for 48 h followed by H2DCFDA staining. Fluorescent intensities were measured at 488 nm/522 nm (Ex/Em wavelengths) by the Beckman flow cytometer. (i) Hitograms of the ROS generation measured for (A) N-acetylcysteine treated (-ve control), (B) 50 μM H2O2 treated (+ve control), (C) and (D) valrubicin treated samples at its 2x IC50 and IC50 dose, respectively; (E) and (F) ciclesonide treated samples at its 2xIC50 and IC50 dose, respectively. (ii) Bar graphs representing the mean ± SEM of fluorescence intensities of the ROS-positive cells from three independent experiments. The significance level compared to the -ve control was calculated by the One-way ANOVA test using GraphPad Prism5 and denoted by *P < 0.05, **P < 0.01, and ***P ≤ 0.001.
I(C)].

Similarly, in valrubicin-treated cells, around 34.81% and 45.14% of the cells were in the G0/G1 phase for IC50 and 2x IC50 doses, respectively [Figure 5.3 I (D) and I(E)]. Moreover, a slight increase in the percentage of SubG1 cells was observed in drug-treated parasites, with no significant changes in the S phase. In the G2/M phase, the percentage of cells was significantly decreased upon drug treatment. The results suggest that both drugs are arresting the cells at the G0/G1 phase and the increase in the subG1 phase indicates apoptosis-like events for the cells.

5.3.4 Drug-induced caspase activation leading to the DNA laddering.

The DNA laddering study on drug-induced *Leishmania* parasites provides crucial insights into the mechanisms of the parasite's death and highlights apoptosis-like characteristics induced by the drugs. DNA fragmentations, typically short DNA-ladders observed in gel electrophoresis, serve as a biochemical indicator of apoptosis, primarily mediated by caspase-activated DNase (CAD), which cleaves DNA at internucleosomal sites (Kitazumi & Tsukahara, 2011)(Ghosh & Roth, 2014). In *Leishmania* promastigotes, apoptosis may proceed via intrinsic or extrinsic pathways, both of which converge on caspase activation and DNA degradation (Sadr et al., 2025). Our study demonstrated that *Leishmania* cells subjected to ciclesonide and

valrubicin displayed light DNA ladderings at both IC_{50} and $2\times IC_{50}$ concentrations, whereas untreated controls exhibited no such patterns (Figure 5.4). These results were similar to the H_2O_2-treated positive controls, suggesting that drug-induced stress activates apoptotic pathways in the parasites, probably through the activation of endogenous nucleases. This observation aligns with previous reports indicating that *Leishmania* parasites, despite the absence of

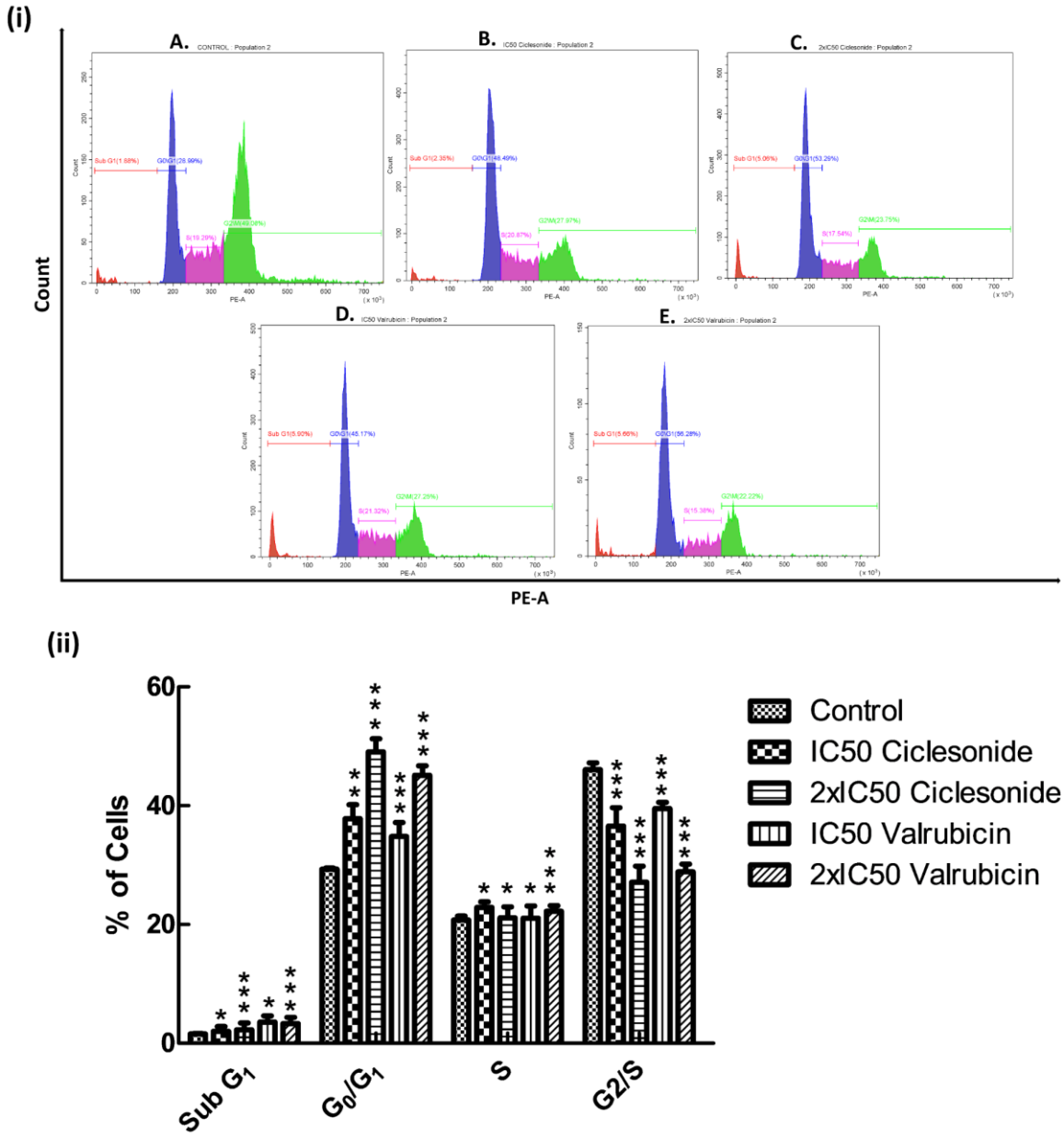


Figure 5.3: Flow cytometric analysis of cell cycle in drug-treated *Leishmania donovani* promastigotes. Parasites were incubated with IC50 and 2xIC50 concentrations of valrubicin and ciclesonide for 48 h, followed by staining with propidium iodide. Fluorescent intensities were

measured by the Beckman flow cytometer. (i) The images show different phases of the cell cycle (A) untreated cells (-ve control), (B) IC50 and (C) 2xIC50 concentrations of ciclesonide-treated samples, respectively, and (D) IC50 and (E) 2xIC50 concentrations of valrubicin-treated samples, respectively. (ii) Bar graph representing the mean ± SEM of intensities from three independent experiments, demonstrating changes in cell cycle populations upon drug treatment. In the drug-treated samples, the number of cells in sub-G1 and G0/G1 phases was significantly increased in a dose-dependent fashion when compared to the control. The significance levels compared to the control were determined by the One-way ANOVA test using GraphPad Prism5 (trial version) and denoted by *P < 0.05, **P < 0.01, and ***P ≤ 0.001.

canonical caspases, demonstrate apoptosis-like programmed cell death, marked by DNA fragmentation, phosphatidylserine externalisation, and mitochondrial depolarisation (Zangger et al., 2002a).

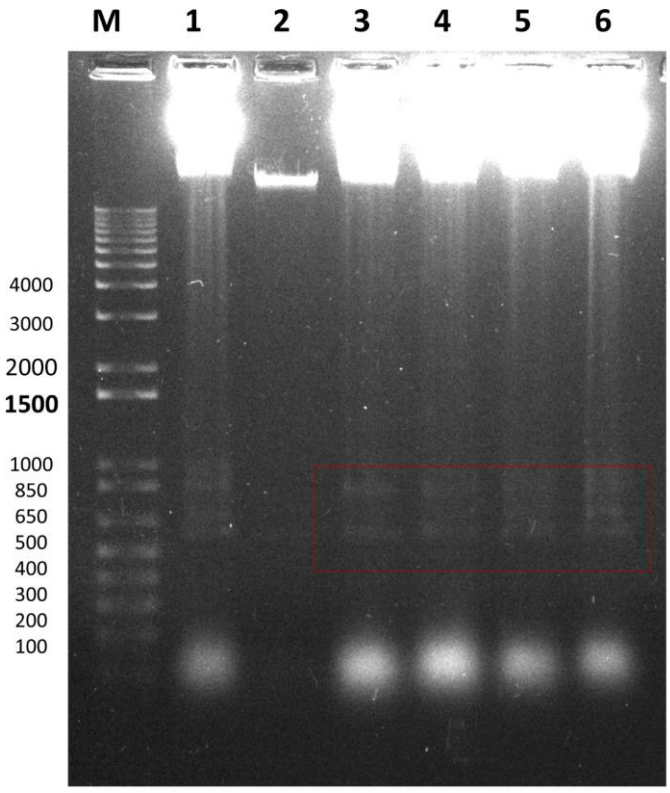


Figure 5.4: DNA fragmentation assay on drug-treated *Leishmania donovani* promastigotes. Genomic DNA fragmentations were analysed where, Lane 1 represents 1 kb + DNA ladder, Lane 2 shows doses of Miltefosine treated sample (+ve control), Lane 3 shows untreated samples, Lane 4 and 5 displays IC50 and 2x IC50 doses of valrubicin treated samples, respectively, Lanes 6 and 7 represent IC50 and 2x IC50 doses of ciclesonide treated samples, respectively.

5.3.5 Annexin V/PI staining indicates apoptosis-like events

The Annexin V/PI dual staining assay, carried out by flow cytometric analysis, is a commonly utilised technique for examining cell death, specifically in differentiating between apoptotic and necrotic cell populations(Lakshmanan & Batra, 2013). The assay relies on two principal physiological indicators: the externalisation of phosphatidylserine (PS) and the compromise of plasma membrane integrity. During early apoptosis, phosphatidylserine translocates from the inner to the outer leaflet of the plasma membrane, allowing preferential binding by FITC-conjugated Annexin V. Conversely, PI is impermeable to membranes and exclusively labels cells exhibiting compromised membrane integrity, indicative of late apoptosis or necrosis. The integration of Annexin V and PI facilitates the categorisation of cell populations into viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) subtypes, thereby offering mechanistic insights into the mechanisms of cell death following therapeutic interventions.

In our study, Miltefosine-treated cells were taken as positive controls, as it has been reported to induce apoptosis in *Leishmania* parasites (Paris et al., 2004). Miltefosine at its IC50 dose, exhibited approximately 38% and 7% early and late apoptotic cells, respectively, which increased to 40% and 26% at the 2xIC50 dose (Figure 5.5). In IC50 valrubicin-treated cells, 27% and 49% of cells were in early and late apoptosis, while at 2x IC50, these values shifted to 5% and 77%, respectively. Notably, at the 2xIC50 dose of valrubicin, 15% of the cells were necrotic, whereas the compound ciclesonide showed minimal induction of apoptosis, with only 19% and 1% of cells in

the early and late apoptosis, respectively, even at the 2xIC50 dose. These findings suggest the significant apoptotic impact of our drug valrubicin on the parasites, while ciclesonide exhibited a substantially lower apoptotic response in the parasites.

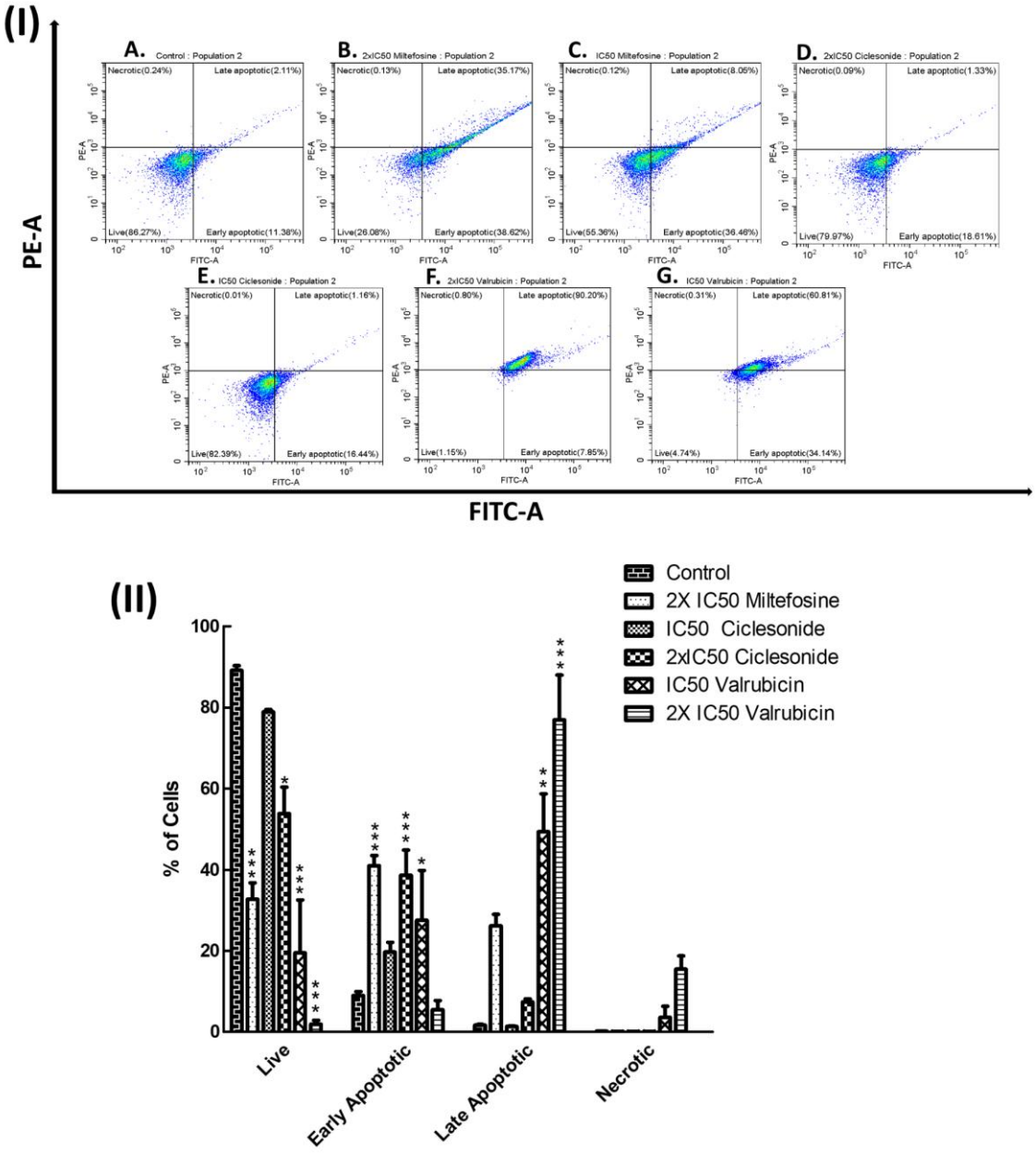


Figure 5.5: Apoptosis and genomic DNA fragmentation assay on drug-treated *Leishmania donovani* promastigotes. Promastigotes were subjected to FITC-conjugated annexin V/PI staining after treatment with Miltefosine, valrubicin, and ciclesonide at various concentrations for 48 h. Flow cytometric analysis was performed to assess apoptosis. (I) Dot plots illustrating different stages of apoptosis in untreated sample (top left panel), Samples treated with 2xIC50 of Miltefosine (top middle panel) Samples treated with ciclesonide at its IC50 (Top left panel), and 2xIC50 doses (Bottom left), and samples treated with valrubicin at its IC50 and 2xIC50 doses (Bottom middle and

bottom right, respectively). FITC-A shows annexin binding, while PE-A shows propidium iodide (PI) staining. (II) The bar graph represents the mean± SEM of percentage distributions of cells from three independent experiments. Significance levels compared to the untreated control are indicated as *P < 0.05, **P < 0.01, and ***P < 0.001.

5.4 Discussion

Understanding the mechanisms of drug-induced cellular death in *Leishmania* parasites is essential for the rational development of effective and safer antileishmanial compounds. This study evaluated the cellular and metabolic responses of *L. donovani* promastigotes to two promising compounds—valrubicin and ciclesonide—identified by in vitro screening. Our findings collectively indicate that these chemicals elicit diverse levels of apoptosis-like characteristics in the parasite, marked by the generation of reactive oxygen species (ROS), cell cycle arrest, DNA fragmentation, and phosphatidylserine externalisation.

The morphological changes demonstrated in scanning electron microscope (SEM) images were among the initial signs of drug-induced stress (Araújo et al., 2011). Untreated promastigotes had the characteristic elongated morphology and undamaged flagella, while treatment with valrubicin and ciclesonide resulted in significant morphological abnormalities. Cells treated with valrubicin displayed significant structural deformities and loss of flagellar integrity, reflecting cytoskeletal disorganisation and reduced cellular damage. This morphological degradation corresponds with prior findings indicating that apoptotic cell death in trypanosomatids frequently involves cell shrinkage, membrane blebbing, and flagellar loss.

Oxidative stress is an important indication of drugs' effectiveness, especially in the context of targeting the parasite's antioxidant defence system. Our ROS study

indicated a distinct, dose-dependent elevation in intracellular ROS levels after treatment with both compounds, with valrubicin exhibiting a more prominent induction than ciclesonide. These findings align with our original hypothesis that the disturbance of redox homeostasis, particularly through the alteration of the glutathione/trypanothione system, may result in mitochondrial malfunction and the initiation of subsequent apoptotic pathways (González-Montero et al., 2024;Sen et al., 2004). Since both compounds inhibit the glutathione synthetase enzyme and which results in the inhibition of the parasitic growth in a dose-dependent manner, these findings indicate that the detected oxidative stress is a direct result of compromised redox equilibrium in the parasite. The increase of reactive oxygen species (ROS) is linked to initiating apoptosis-like processes in *Leishmania*, which might involve the release of mitochondrial factors such as Endonuclease G (EndoG) and apoptosis-inducing factor (AIF), which facilitate chromatin condensation and DNA destruction (P. Das et al., 2021).

Consistent with the intracellular ROS elevation upon treatment with valrubicin and ciclesonide, both these compounds also arrested the parasite's cell-cycle progression at the G0/G1 phase, increasing the sub-G1 population. Cell cycle progression is a normal phenomenon of healthy cells that facilitates DNA repair or activates regulated death pathways in case of irreparable damage (Thangaraj et al., 2019). In contrast, the cell cycle arrest at the G0/G1 phase is linked to cells undergoing stress-induced apoptosis (Liu et al., 2003; Song et al., 2017). The increase of the sub-G1 population further correlates with the incidence of DNA -fragmentation, a characteristic of apoptotic induction (Kajstura et al., 2007a, 2007b). Thus, this result shows that the compounds, valrubicin and ciclesonide, were able to disrupt the parasite's redox balance, leading to oxidative stress.

To further investigate the occurrence of DNA fragmentation, we performed a DNA-laddering assay by gel electrophoresis, where light bands of DNA laddering were observed, further solidifying the hypothesis. It is also important to note that *Leishmania* parasites do not possess traditional caspases and instead use alternative nucleases like EndoG for DNA fragmentation (Zangger et al., 2002a) (Ghosh & Roth, 2014). Therefore, the induction of intra-nucleosomal DNA cleavage might be the result of drug-induced stress activation of caspase-independent programmed cell death.

The final evidence of apoptosis-like cell death was achieved from flow cytometric analysis after Annexin V/PI staining. The quantitative analysis of apoptotic cell population using Annexin V/PI dual staining is a robust method that relies on the detection of phosphatidylserine exposure on the outer leaflet of the plasma membrane and assessing plasma membrane integrity (Alshehade et al., 2024; Pietkiewicz et al., 2015). Our analyses show that valrubicin can induce apoptosis with a significant increase in the early and late apoptotic cell populations in a dose-dependent manner. However, ciclesonide can induce apoptosis at a low intensity, indicating a less potent or alternative mode of action (P. Das et al., 2021). Therefore, the secondary mode of action of ciclesonide, in addition to the inhibition of glutathione synthetase, cannot be ruled out. These results therefore demonstrate valrubicin's ability to trigger apoptosis with different apoptotic markers, hence strengthening its potential as an antileishmanial drug. Also, the small percentage of cells shifting to the necrotic populations while treating at 2xIC50 concentration may indicate sustained or excessive reactive oxygen species (ROS) accumulation in the *Leishmania* might have exceeded the apoptotic threshold, leading to membrane damage and unregulated cell death, a phenomenon documented in other research on oxidative stress-induced toxicity (Paris et al., 2004;Villalpando-Rodriguez & Gibson, 2021).

The comparative examination of valrubicin and ciclesonide indicates that both drugs disrupt redox equilibrium and produce growth arrest; however, valrubicin is markedly more effective in inducing programmed cell death. These findings are significant since they demonstrate that valrubicin and cilcesonide exert their antiparasitic effects through pathways that differ from those of current chemotherapeutics. Therefore, these drugs' ability to trigger late apoptosis and necrosis by disrupting redox balance may be effective against drug-resistant strains of *Leishmania* parasites.

These findings highlight the complexity of cell death pathways in *Leishmania*, which, despite the absence of classical apoptotic mechanisms(Basmaciyan et al., 2018),(Zangger et al., 2002b), may engage in precisely regulated death programs in response to pharmacological stress. The activation of these pathways by valrubicin and, to a lesser degree, by ciclesonide illustrates the potential to exploit parasite-specific weaknesses for targeted elimination. Understanding these molecular aspects facilitates the validation of therapeutic targets and establishes a foundation for formulating combination therapies that synergistically induce parasite death pathways while reducing host damage. This study conclusively demonstrates that valrubicin produces apoptosis-like cell death in *Leishmania donovani* via oxidative stress, cell cycle arrest, and DNA fragmentation. Ciclesonide, while less effective in triggering apoptosis, also impairs parasite function, indicating a potential alternate or supplementary mechanism of action. These findings enhance our comprehension of drug-induced cellular apoptosis in *Leishmania* and validate the further preclinical advancement of both valrubicin and ciclesonide as viable candidates for antileishmanial treatment.