CHAPTER 4

Combating Miltefosine Unresponsiveness in *Leishmania donovani*: Synergistic Efficacy of Veratramine Co-Treatment

Abstract*

The emergence of drug-resistant *Leishmania donovani* poses a critical challenge to the treatment of visceral leishmaniasis (VL). This study evaluates the inhibitory activity of Veratramine, a steroidal alkaloid, against miltefosine-unresponsive *L. donovani* and explores its potential in combination therapy with Miltefosine. Veratramine demonstrated inhibitory activity against miltefosine-unresponsive *L. donovani*, with an IC_{50} of 28.81±0.20 µM, compared with 12.46 ± 2.28 µM in the sensitive strain, indicating partial unresponsiveness. Miltefosine alone exhibited similar inhibitory potency (IC_{50} = 26.82±0.52 µM), but in combination with sub-IC_{50} Veratramine (12 µM), Miltefosine's IC_{50} decreased sharply to 12.22±0.40 µM. Checkerboard assays with different combinations of Miltefosine and Veratramine confirmed the strong synergy even at lower concentrations, and a combination of 1.7 µM Veratramine and 1.8 µM Miltefosine shows 50% parasitic death, suggesting the potential to use reduced dosages for both drugs. Scanning electron microscopy revealed significant morphological abnormalities following mono- and combination therapies, consistent with cellular stress and cell death pathways. Biochemical assays indicated that while miltefosine monotherapy induced apoptosis, the Veratramine-combination test group prompted minimal apoptosis and necrosis, but increased production of autophagic vacuoles and acidic organelles. These findings suggest that the Miltefosine-Veratramine combination, owing to its antileishmanial activity, is mediated by autophagy, and its combination with Miltefosine bypasses unresponsiveness to some extent through non-apoptotic mechanisms. The synergistic effect likely results from mutual targeting of parasite survival pathways, providing a promising strategy to manage drug-resistant VL.

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* Part of the work is submitted for publication

4.1 Introduction

Miltefosine, an alkyl phosphocholine drug, represents a breakthrough in the treatment of visceral leishmaniasis (VL) as the first oral therapeutic with demonstrated efficacy against *Leishmania donovani* infections. However, the increasing reports of miltefosine unresponsiveness and clinical cases involving miltefosine-unresponsive *L. donovani* strains have raised considerable concerns regarding the long-term sustainability of this drug in endemic regions (Chakravarty and Sundar, 2019b).

The development of miltefosine unresponsiveness in *L. donovani* is complex and appears to be multifactorial. A key feature driving unresponsiveness is the diminished intracellular concentration of miltefosine achieved within the parasite. This reduction primarily results from defects in parasite membrane transport systems. Specifically, mutations or downregulation of the miltefosine transporter complex, composed of the *LdMT* gene encoding a phospholipid translocase and its accessory subunit *LdRos3*, significantly impair drug uptake (Pérez-Victoria et al., 2006a). Consequently, resistant parasites exhibit reduced drug accumulation, limiting miltefosine's cytotoxic actions (Chakravarty and Sundar, 2010; Croft et al., 2006; Papadopoulou, 1993).

In addition to faulty uptake, increased activity of ATP-binding cassette (ABC) transporters facilitates enhanced drug efflux, further lowering intracellular miltefosine concentration. This dual mechanism, impaired influx coupled with augmented efflux, underpins the unresponsive phenotype (Pérez-Victoria et al., 2006a).

Isolates from patients who relapse after miltefosine treatment, as well as laboratory-induced resistant strains, have been shown to possess distinctive features contributing to therapeutic failure. These strains typically:

- Demonstrate significantly reduced miltefosine accumulation due to altered transporter expression or function.
- Exhibit alterations in mitochondrial function and stress response pathways, which protect the parasite from miltefosine-induced programmed cell death (apoptosis-like) typically observed in the drug-sensitive strains. For instance, elevated expression of Fe-superoxide dismutase A (FeSODA) and the NAD-dependent deacetylase SIR2 enhances unresponsiveness by combating oxidative stress and preventing apoptosis (Chakravarty and Sundar, 2019b).
- Show metabolic reprogramming, including increased thiol levels and modifications in glycolytic and lipid metabolism enzymes, supporting survival during drug exposure. Upregulation of lipase precursor proteins may also confer an advantage by facilitating energy utilisation under stress.
- Retain or even augment their virulence potential by enhanced metacyclogenesis and infectivity towards host macrophages, which likely contributes to persistent infection and higher relapse rates observed clinically (Sundar and Rai, 2002).

The emergence of miltefosine-unresponsive *L. donovani* strains threatens the efficacy of one of the few oral antileishmanial treatments available, posing major challenges to VL control efforts in endemic countries like India and Nepal. Frequent relapses following miltefosine therapy compromise treatment outcomes and increase disease burden.

Current diagnostic methods lack rapid and reliable molecular tests to detect miltefosine unresponsiveness in clinical settings, highlighting the need for robust surveillance systems. Judicious use of miltefosine, preferably in combination therapies and continuous monitoring through molecular markers of resistance, is a recommended strategy to mitigate the rise and spread of resistant parasites.

Miltefosine unresponsiveness in *Leishmania donovani* primarily arises due to mutations and altered expression in the miltefosine transporter complex (*LdMT*/*LdRos3*), coupled with increased drug efflux mediated by ABC transporters. Miltefosine-unresponsive strains evade drug-induced apoptosis-like death through enhanced antioxidant defences and metabolic adaptations, sustaining infectivity and contributing to relapse. Addressing these mechanisms through improved diagnostics, combination treatments, and novel drug development is critical for sustaining the gains achieved in visceral leishmaniasis control (Pérez-Victoria et al., 2006b; Seifert et al., 2007).

4.2 Materials and methods

4.2.1 Biological studies

The preparation of drugs involved dissolving miltefosine in PBS and veratramine in DMSO. The working concentration was made in M199 media for treatment. 100µM H_2O_2 (dissolved in ultrapure water) was used as a positive control, and 200nM Rapamycin was used as an autophagy inducer. The untreated promastigote cells were used as a negative control. The flowcytometric experiments were performed using a CytoFlex flow cytometer (Beckman Coulter), and the data were analysed using the CytExpert v 2.4.0.28 software. At least 10,000 events were recorded for each experiment, and 1000 events were displayed. The fluorescence imaging was executed using an Olympus BX 63 fluorescence microscope. The images were analysed using ImageJ 1.54 d software.

4.2.2 Cultures and Chemicals

The Miltefosine-unresponsive *Leishmania donovani* strain used for this study (Sample ID:1119) was kindly donated by Prof. Shyam Sundar and Dr. Rajiv Kumar from IMS, BHU.

The primary culture was isolated from a 10-year-old male from Bihar in 2010. The promastigotes were cultured in M199 media containing 10% FBS at 25ºC in the dark. The entire reagents and chemicals used for this study were of the highest grade for cell culture and were procured from Thermofisher Scientific and Sigma-Aldrich.

4.2.3 Estimation of the inhibitory effect of test compounds on miltefosine-unresponsive *Leishmania donovani*

The inhibitory effect of miltefosine, veratramine and their combination was estimated to determine their efficacy on the miltefosine-unresponsive *Leishmania donovani* strain using the MTT assay. The IC_{50} concentrations were determined as described in previous studies. The 96-well plate was used for the assay with a total volume of 200µl. The wells were first loaded with serial dilutions of the test compounds and later seeded with 5×10^5 cells/ml promastigotes. The plate was incubated for 48hrs at 25ºC in the dark. Wells without any drug were considered as a negative control, and wells with only M199 media were considered as a blank to eliminate any background noise. The MTT reagent (0.5 mg/ml) was added to each well and incubated for 4 hours in the dark at 25ºC. The formazan crystals produced were dissolved in 150µl DMSO, and the absorbance was estimated at 570nm using a BioTek Synergy HT microtiter plate reader. The inhibitory concentrations were calculated from the dose-response curve.

4.2.4 Determination of ultrastructural changes after drug treatment

The changes in morphology of miltefosine-unresponsive *Leishmania donovani* after drug treatment were analysed using Scanning Electron Microscopy (BT-SEM (JSM 6000 PLUS). The promastigotes (2×10^6 cells/ml) were seeded in a 24-well plate along with test compounds and incubated for 48 hours in the dark at 25ºC. The untreated cells were considered as the negative control, and the cells treated with 500µM H_2O_2 were considered as the positive control. After treatment, the cells were washed twice with phosphate buffer (PB) and fixed in

2.5% glutaraldehyde for 1h at room temperature (RT). The cells were then washed once with PB and twice with ultrapure water. The cells were then serially dehydrated using ethanol, mounted on a coverslip and observed under SEM.

4.2.5 Evaluation of phosphatidylserine externalisation

During apoptosis, a process called membrane flipping, specifically phosphatidylserine (PS), occurs, where the PS from the inner side is exposed to the outer side. This was determined by staining the cells with annexin, and to distinguish from necrotic cells, it is co-stained with PI. The assay was conducted using FITC Annexin V/Dead cell Apoptosis kit (Invitrogen) according to the manufacturer's instructions. A total of 2×10^6 cells/ml were seeded into a 24-well plate along with the test compounds. The untreated promastigotes and 500µM H_2O_2 treated promastigotes were used as the positive and the negative controls, respectively. After 48 hours of incubation in the dark at 25ºC, the cells were washed twice with cold PBS (Phosphate Buffer Saline). The cells were then resuspended in 100µl of 1X annexin-binding buffer along with the dyes and allowed to incubate at RT for 15 minutes. Later, 400µl of 1X annexin-binding buffer was added, mixed gently, and analysed in a flow cytometer immediately.

4.2.6 Detection of acidic organelle production

The dual staining nature of Acridine orange (AO) has been used to detect the induction of acidic organelles after treatment with test compounds. AO binds to nucleic acids and acidic organelles and emits green and red fluorescence, respectively. About 2×10^6 cells/ml along with test compounds and controls were incubated for 48 hours in the dark at 25ºC. The cells were then washed twice with PBS and stained with AO (5 µg/mL) for 15 minutes in the dark at RT. Later, the cells were washed twice with PBS, subsequently fixed with 4% PFA (Paraformaldehyde) and observed under a fluorescence microscope.

4.2.7 Detection of Autophagic vacuole production

The formation of double-membrane vesicles is observed in cells during the onset of autophagy. These vesicles are autophagosomes, which fuse with lysosomes to form autophagolysosomes. These autophagic vacuoles can be detected using the CYTO-ID Autophagy detection kit (Enzi Life Science). The 24-well plate was seeded with 2×10^6 cells/ml along with the test compounds, and the analysis was done after incubation for 48 hours at 25ºC in the dark. The untreated cells were considered as the negative control, and 200nM Rapamycin (autophagy inducer) treated cells were considered as the positive control. The cells were washed twice with 1X assay buffer and stained with Hoechst 33342 and CYTO-ID for 30 minutes at RT. The promastigotes were washed with 1X assay buffer and fixed with 4% PFA. The cells were observed under a fluorescence microscope to detect the presence of autophagic vacuoles.

4.2.8 Statistical analysis

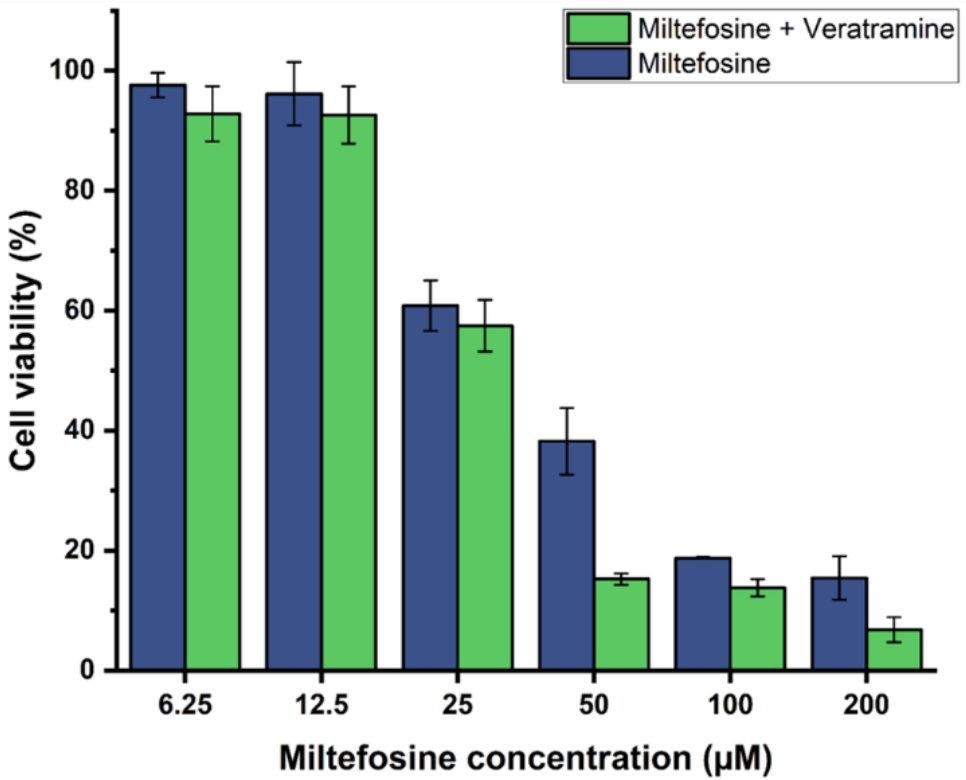
All the samples were analysed by using at least two independent experiments. In flow cytometric experiments, values were represented as the percentage positive promastigotes for each marker from the total promastigote population. The OriginPro 2025b software was used for plotting all data points, and the different significance levels were calculated using a two-way analysis of variance test (where ** $p < 0.005$ and *$p < 0.05$). The fluorescence intensity of fluorescence microscopy data was determined using ImageJ 1.54 d software.

4.3 Results

4.3.1 Inhibitory analysis of selected drug compounds on miltefosine-unresponsive *Leishmania donovani*

The previous studies showed that the steroidal alkaloid compound, Veratramine, exhibited anti-leishmanial activity through a mechanism that involves autophagy. The IC_{50} of Veratramine in *Leishmania donovani* (AG83) promastigotes was observed as 12µM. In this study, the inhibitory effect of Veratramine on miltefosine-unresponsive *Leishmania donovani* was evaluated, and the IC_{50} concentration was estimated to be 28.81 ± 0.20 µM. The inhibitory concentration of Miltefosine alone and the inhibitory concentration of Miltefosine in the presence of 12µM Veratramine on miltefosine-unresponsive *Leishmania donovani* were also determined. The IC_{50} concentration of Miltefosine monotherapy was calculated as 26.82 ± 0.52 µM, and the IC_{50} concentration of Miltefosine in combination with 12µM Veratramine was calculated as 12.22 ± 0.40 µM (Figure 23). This data suggests that Veratramine reduces the Miltefosine concentration required to exhibit the same inhibitory effect as Miltefosine monotherapy on miltefosine-unresponsive *Leishmania donovani*. This initial experiment indicated that there is some synergistic effect of Veratramine- Miltefosine combination therapy on the unresponsive promastigotes. Through the Checkerboard method, it was determined that about 1.7 µM of Veratramine and 1.8 µM of Miltefosine combination exhibited a 50% inhibitory effect on the unresponsive promastigotes after 48 hours of treatment (Table 10).

(A)



(B)

Examined Drugs	IC_{50} (μM)
Miltefosine	26.82 ± 0.52
Veratramine	28.81 ± 0.20
Miltefosine (in the presence of 12μM Veratramine)	12.22 ± 0.40

Figure 23: (A) Comparative analysis of the inhibitory effects of miltefosine monotherapy versus miltefosine co-administered with veratramine (12 μM) on miltefosine-unresponsive *Leishmania donovani* promastigotes. (B) Half-maximal inhibitory concentration (IC_{50}) values for miltefosine, veratramine, and their combination (miltefosine + veratramine at 12 μM) against miltefosine-unresponsive *L. donovani* promastigotes.

Table 10: Inhibitory activity of the drug combination against promastigotes of the miltefosine-unresponsive *Leishmania donovani* strain following 48 hours of incubation

Veratramine concentration (µM)	Miltefosine concentration (µM)					
	28.8	14.4	7.2	3.6	1.78	0.89
26.8	67.46 ± 2.37	66.59 ± 1.26	64.38 ± 1.73	64.3 ± 1.02	65.88 ± 0.71	65.25 ± 1.34
13.4	62.24 ± 2.76	61.69 ± 2.05	60.43 ± 0.79	60.98 ± 0.71	59.08 ± 1.81	60.03 ± 0.71
6.7	62.72 ± 1.81	60.11 ± 0.31	58.21 ± 0.47	56.79 ± 0.94	54.26 ± 1.26	56 ± 0.94
3.35	62.64 ± 1.73	59.87 ± 0.71	54.34 ± 1.34	54.97 ± 0.07	52.29 ± 0.86	51.5 ± 3.87
1.66	62.09 ± 2.76	59.32 ± 1.1	51.5 ± 0.55	52.68 ± 0.31	50.15 ± 0.63	52.6 ± 3.39
0.83	59.64 ± 0.79	60.51 ± 2.13	55.61 ± 0.39	56.32 ± 0.63	53.79 ± 2.21	52.21 ± 1.1

4.3.2 Analysis of the microstructural alterations

The modifications in the cell morphology of the promastigotes after 48 hours of treatment with test compounds were observed under SEM. It was observed that there is a notable alteration in the cell morphology as compared to the untreated cells after 48 hours of treatment with the test drugs. The promastigotes treated with the IC_{50} concentration of Miltefosine (27µM), Veratramine (29µM) and their combination (1.7 µM Veratramine and 1.8 µM Miltefosine) indicated structural abnormalities like cell swelling, cell rounding, flagella loss, flagella deformation and cellular distortion (Figure 24).

4.3.3 Assessment of Phosphatidylserine externalisation

The PS externalisation of the cell membrane is associated with the induction of apoptosis. The annexin V is a protein that binds specifically to the PS. This binding property is used to identify and quantify the apoptotic cells. The cells treated with 500µM H_2O_2, IC_{50} concentration of Veratramine (~29µM) and IC_{50} concentration of Miltefosine (~27µM) indicated more than 50% apoptotic population. The cells treated with the combination (1.7 µM Veratramine and 1.8 µM Miltefosine), however, did not exhibit any significant increase in apoptotic population. The promastigotes treated with the test compounds displayed some necrotic population, with miltefosine monotherapy inducing the maximum necrosis (~ 15%), followed by Veratramine and H_2O_2, and the combination therapy inducing the least necrosis (Figure 25).

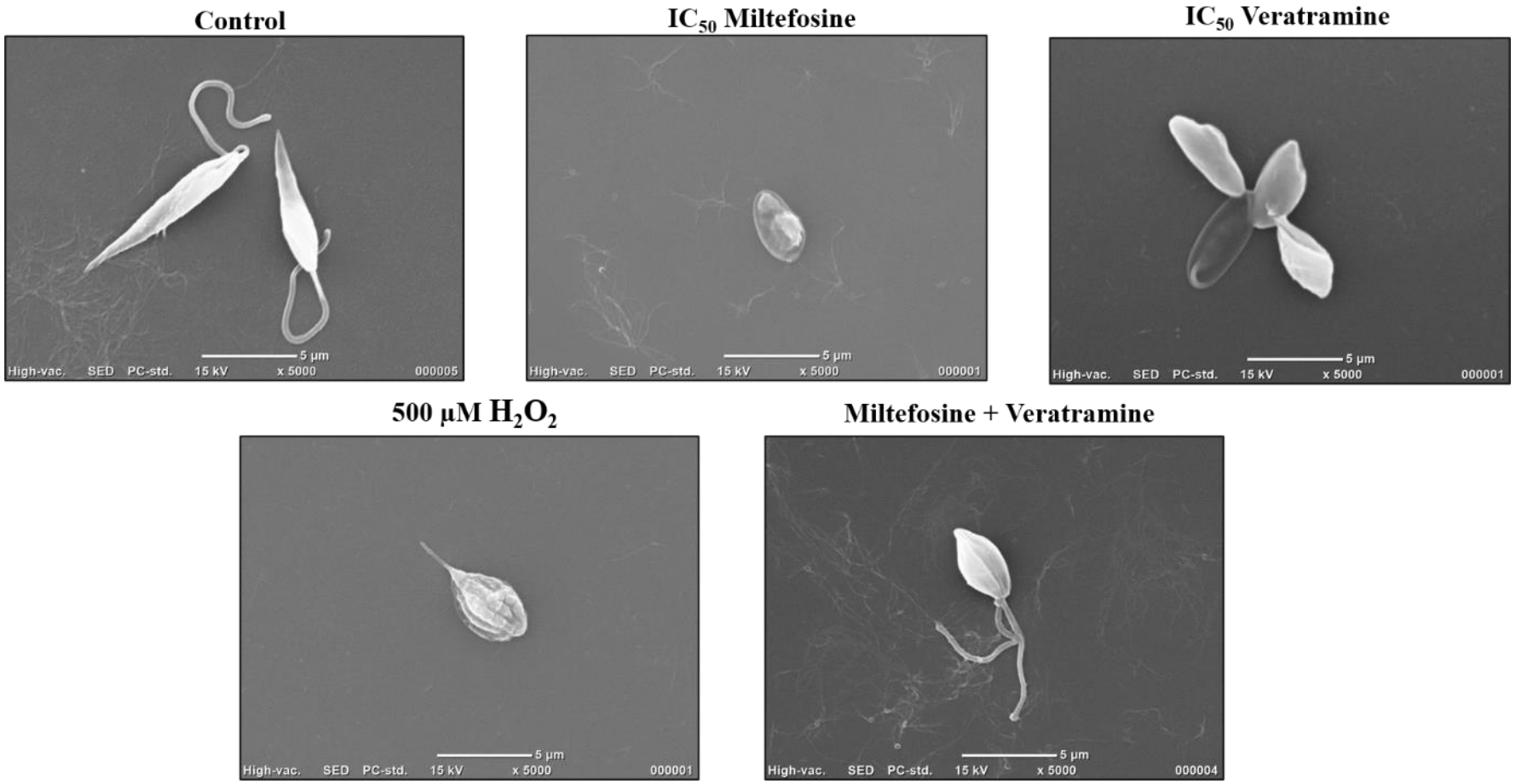


Figure 24: Ultrastructural alterations in Miltefosine-unresponsive *L. donovani* promastigotes were characterised using scanning electron microscopy (SEM). Notable morphological changes were observed following 48 hours of treatment. Promastigotes treated with 500 µM hydrogen peroxide (H_2O_2) served as the positive control, while untreated promastigotes were used as the negative control.

4.3.4 Analysis of acidic organelles production

The stress-induced acidic organelle production in the miltefosine-unresponsive *Leishmania donovani* after 48 hours of treatment with the test compounds was examined by staining the cells with AO. The AO binds to the membrane-bound acidic organelles and emits red fluorescence at acidic pH. The promastigotes treated with Veratramine displayed the highest stress-induced acidic organelle production, followed by the combination therapy, and the lowest production in Miltefosine monotherapy (Figure 26 (A)). The cells treated with Veratramine monotherapy and Miltefosine-Veratramine combination therapy indicated a significantly high percentage of stress-induced acidic organelle production as compared to the positive control, considering promastigotes treated with 500µM H_2O_2 producing 100% red fluorescence intensity (Figure 26 (B)).

4.3.5 Detection of Autophagic vacuole production

The exposure of cells to chemical stress leads to damaged and dysfunctional cellular components. This leads to an onset of cellular events that direct autophagic organelle production, which is the cellular defence mechanism for cell survival. These vacuoles degrade and recycle the impaired cellular components and restore the cellular functions. As discussed in the previous studies, Veratramine induces autophagic vacuole production in AG83 *Leishmania donovani* promastigotes. To check whether there is a similar effect on the miltefosine-unresponsive *Leishmania donovani* strain, the promastigotes were examined for autophagic vacuole production after 48 hours of treatment with the test compounds using CYTO-ID staining and observation under a fluorescence microscope. The observations indicated that the cells treated with Veratramine monotherapy and Miltefosine-Veratramine combination therapy depict similar results as observed previously (Figure 27 (A)). The Veratramine monotherapy and Miltefosine-Veratramine combination therapy indicated a

significant increase in the autophagic vacuole production compared to the positive control, Rapamycin (Figure 27 (B)).

4.4 Discussion

This study presents a detailed inhibitory analysis of Veratramine, a steroidal alkaloid compound, against the miltefosine-unresponsive *Leishmania donovani* strain, and investigates its potential in combination therapy with Miltefosine. These findings offer critical insights into overcoming drug unresponsiveness in visceral leishmaniasis (VL).

The observed IC_{50} of Veratramine (28.81 ± 0.20 μM) against miltefosine-unresponsive *L. donovani* is over twice that required for the AG83 (sensitive) strain (12 μM), highlighting a degree of cross-resistance, but also demonstrating retained activity. The cross-resistance observed with respect to Veratramine could be explained by the mechanisms that the miltefosine-unresponsive *L. donovani* have adapted at the biochemical and genetic level to develop resistance to Miltefosine. The miltefosine-unresponsiveness is a result of downregulation of protein transporters, leading to reduced drug uptake into the cells, the upregulation of drug efflux transporters, alteration in lipid metabolism, and improved oxidative stress management due to the expression of certain genes that modify oxidative metabolism. These modifications not only influence Miltefosine uptake and effect, but might also have a significant effect on Veratramine, especially the genetic adaptation of the parasite.

Miltefosine, used as monotherapy, showed an IC_{50} of 26.82 ± 0.52 μM. However, when used in combination with a fixed, sub-IC_{50} dose of Veratramine (12 μM), the IC_{50} for miltefosine dropped sharply to 12.22 ± 0.40 μM, indicating a good synergistic effect.

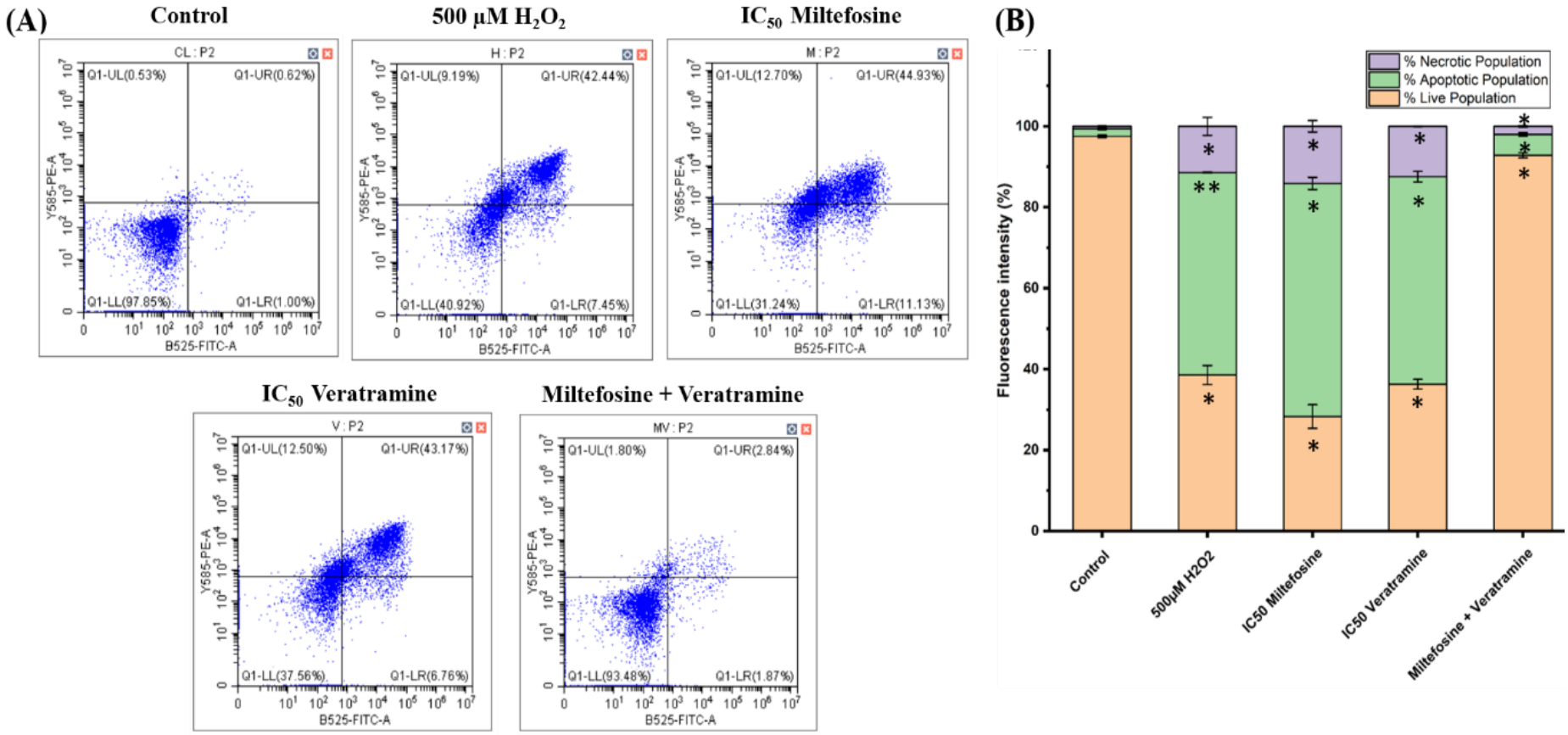


Figure 25: Phosphatidylserine externalisation was assessed using Annexin V–PI dual staining following 48 hours of treatment in a Miltefosine-unresponsive *Leishmania donovani* strain. (A) Representative density plots depict promastigote populations post-treatment with the test compound. (B) A stacked bar graph quantitatively illustrates the distribution of live, early+late apoptotic, and necrotic promastigotes. Treatment with 500 µM hydrogen peroxide (H_2O_2) served as a positive control for apoptosis induction. Significant differences were considered concerning the untreated parasite (control), where $p \leq 0.05$ (*) and $p \leq 0.005$ (**).

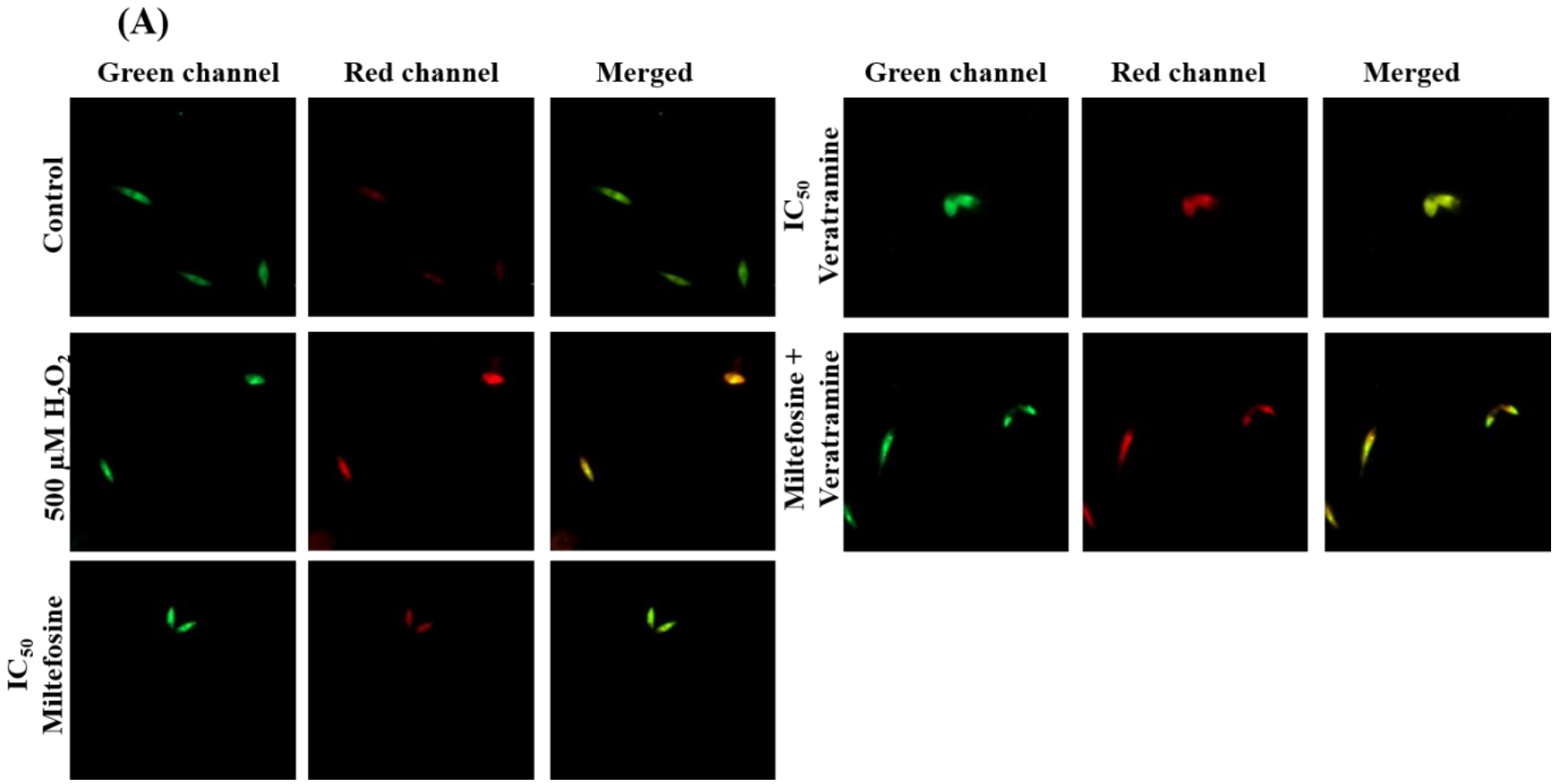


Figure 26 (A): Acridine orange staining reveals induction of acidic organelles following drug treatment. Representative fluorescence microscopy images showing enhanced formation of acidic vacuoles in the *L. donovani* miltefosine-unresponsive strain upon treatment with the test compound. Untreated parasites served as the negative control, while treatment with 500 µM H_2O_2 was used as a positive control.

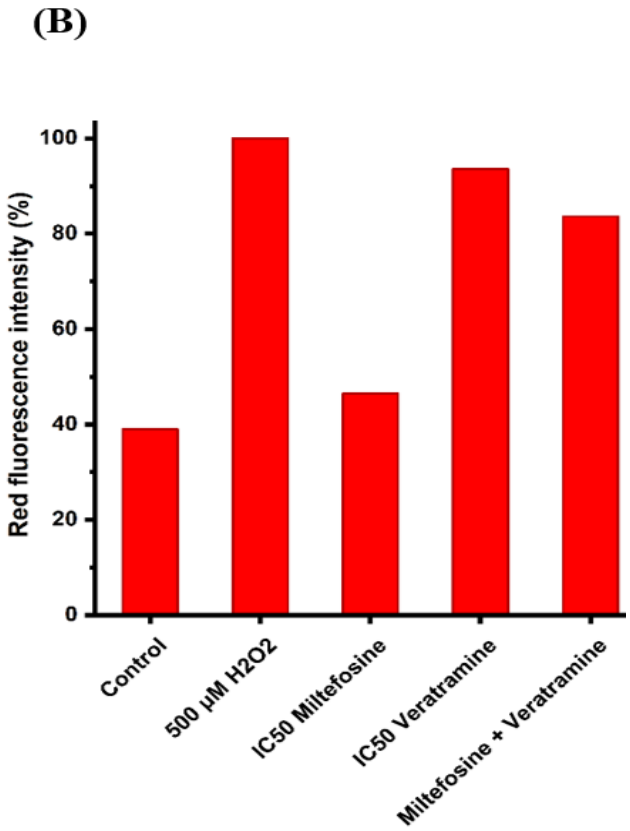


Figure 26 (B): Acridine orange staining reveals induction of acidic organelles following drug treatment. Quantitative analysis of red fluorescence intensity corresponding to acidic organelles detected in the red emission channel. Untreated parasites served as the negative control, while treatment with 500 µM H_2O_2 was used as a positive control. The treatment concentration of 500 µM H_2O_2 was defined as the reference standard (100%) for red fluorescence intensity normalisation.

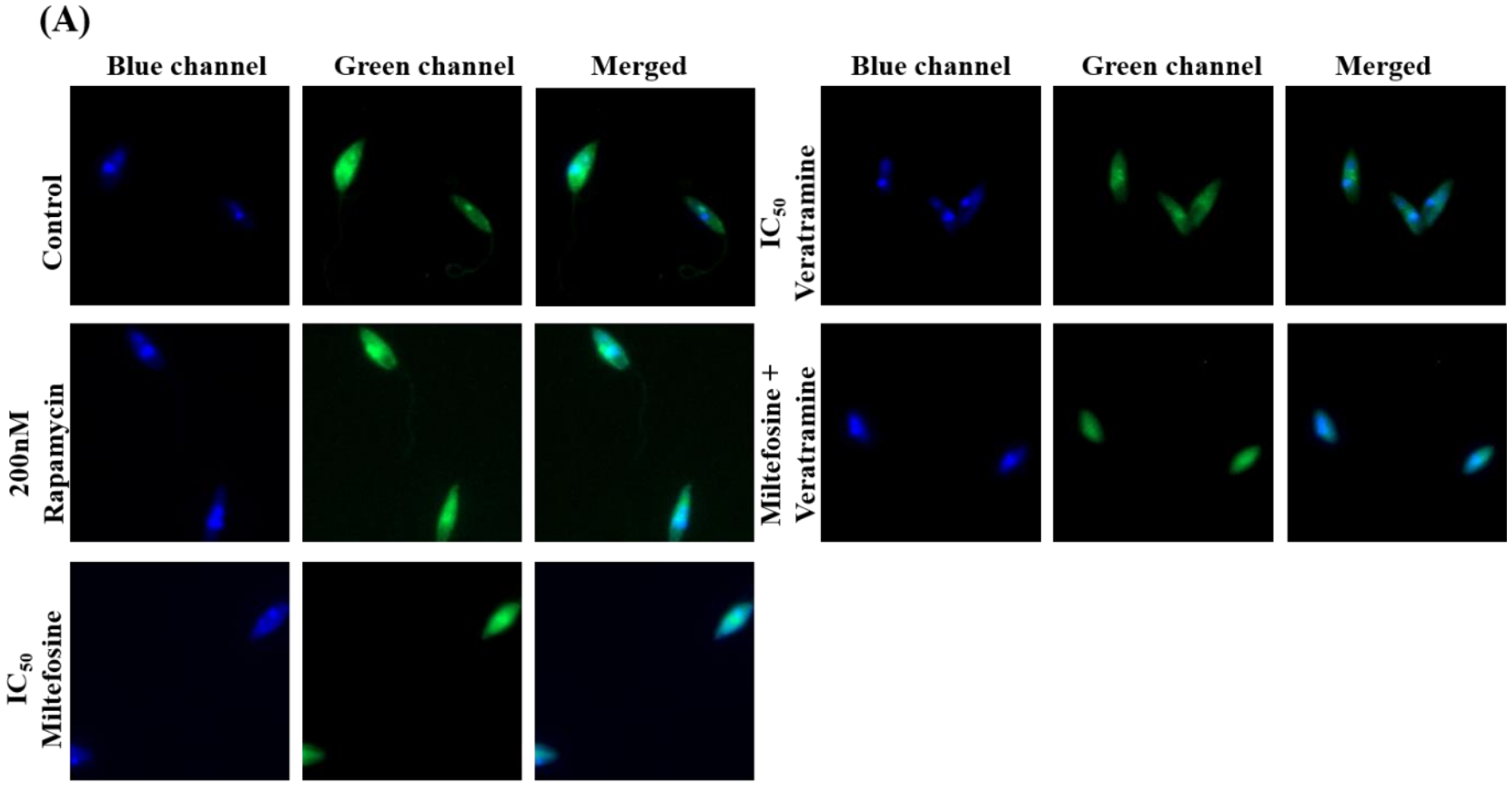


Figure 27 (A): Fluorescence-based assessment of autophagic vacuole accumulation in miltefosine-unresponsive *Leishmania. donovani* promastigotes. Representative fluorescence microscopy images of miltefosine-unresponsive *L. donovani* promastigotes following 48 hours of treatment with the indicated test compounds. Cells were stained with CYTO-ID to visualise autophagic vacuoles and Hoechst 33342 for nuclear staining. Promastigotes treated with rapamycin served as a positive control for autophagy induction, while untreated cells served as the negative control.

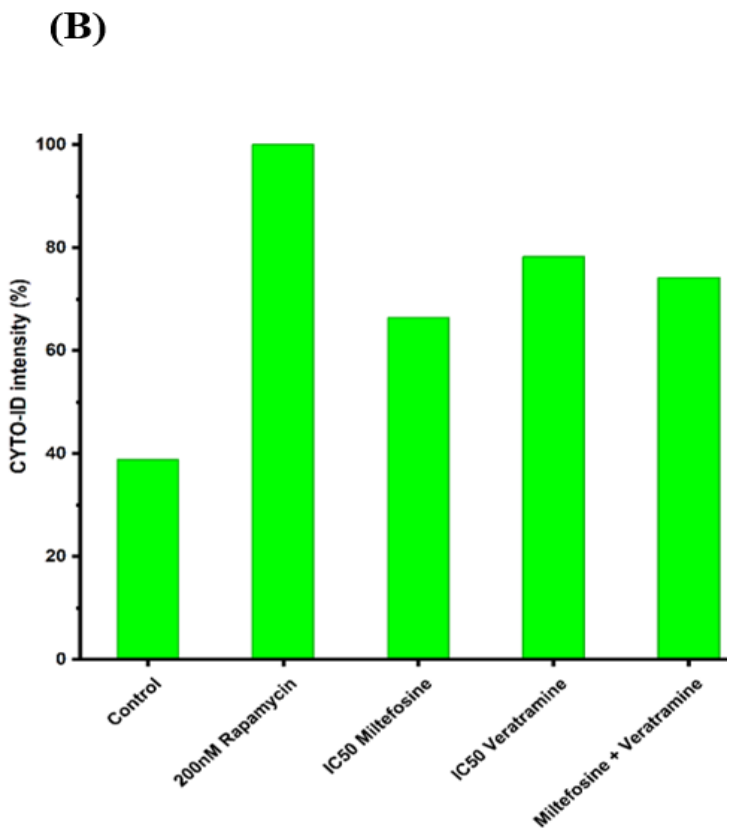


Figure 27 (B): Fluorescence-based assessment of autophagic vacuole accumulation in miltefosine-unresponsive *Leishmania. donovani* promastigotes. Quantitative analysis of relative CYTO-ID fluorescence intensity, representing autophagic vacuole accumulation. Promastigotes treated with rapamycin served as a positive control for autophagy induction, while untreated cells served as the negative control. Fluorescence intensity in rapamycin-treated promastigotes was set as 100% for normalisation across samples.

The checkerboard assay with different concentrations of Veratramine and Miltefosine corroborated this synergy at even lower doses for both compounds (1.7 μM Veratramine and 1.8 μM Miltefosine), achieving 50% inhibition after 48 hours. Such synergy is of particular interest given the challenge of drug unresponsiveness in VL and points to the possibility of using lower doses in combination regimens to overcome unresponsiveness and reduce toxicity.

SEM analysis of treated cells revealed distinct microstructural abnormalities, cell swelling, rounding, flagellar deformation, and cellular distortion across all treatments, but also after combination therapy. These observed changes are consistent with cellular stress and integrity, similar to those previously described in miltefosine-sensitive *Leishmania donovani*. The annexin V assay demonstrated that while Miltefosine monotherapy induced significant phosphatidylserine (PS) externalisation, a marker of apoptosis, the combination therapy did not significantly increase the apoptotic population, and necrosis was also lowest in the combination group. This suggests that the synergy between Veratramine and Miltefosine may involve non-apoptotic mechanisms such as autophagy, consistent with earlier studies which involve autophagy mechanisms. Strangely, Veratramine was also observed to exhibit PS externalisation, which was more than 50% in the miltefosine-unresponsive *Leishmania donovani* strain. This observation was not in congruence with the results evaluated in the miltefosine-sensitive *Leishmania donovani* strain.

Acidic organelle production, assessed by acridine orange (AO) staining, was most pronounced with Veratramine, lesser in combination therapy, and least with Miltefosine. Veratramine-induced stress correlates with the activation of autophagy, a protective mechanism in *Leishmania* previously noted. The combination therapy level of acidic organelle production may reflect the interplay between drug actions, leading to cell death. CYTO-ID staining revealed that both Veratramine and the Miltefosine-Veratramine combination robustly induced autophagic vacuole formation, compared to Rapamycin (an autophagy inducer) as a positive

control. These findings depict the mechanism of Veratramine engaging the autophagy pathway for anti-leishmanial effect and suggest that the efficacy of the combination is mediated by enhanced autophagy. This is especially significant in Miltefosine-unresponsive strains, which are known to evade apoptosis through upregulation of antioxidant defences and metabolic rewiring.

A peculiar apoptotic response was noticed in the miltefosine-unresponsive *Leishmania donovani* strain treated with Veratramine, which was contrary to what we have previously observed in AG83 *Leishmania donovani* strain. Here, in the miltefosine-unresponsive *Leishmania donovani* strain, we could notice that Veratramine shows more than 50% of FITC-positive population, which indicates that it induces apoptosis. Later, when the promastigotes were analysed for autophagy through CYTO-ID staining, Veratramine also exhibits autophagic vacuole formation. Most of the unresponsive strains do not have a complete loss of susceptibility to the drug; instead, multiple mechanisms shift the threshold, and there is no complete loss of drug activity or effectiveness. At higher drug concentrations, the toxic threshold is exceeded, and again the drug is effective (Chakravarty and Sundar, 2010; Croft et al., 2006; Papadopoulou, 1993). In the case of miltefosine, at higher concentrations, secondary nonspecific effects might become dominant. These effects could include other modes of membrane disruption and lipid signalling interference, which can overcome the parasite's defence adaptations. Similarly, the pattern observed with veratramine can be explained by the theory that the higher dose of Veratramine must have resensitised the parasite along with other cellular effect that involves apoptosis-like cell death mechanisms (Pérez-Victoria et al., 2006b; Seifert et al., 2007).

There are many compounds that are reported to exhibit the presence of both autophagy and apoptosis markers of cell death in *Leishmania*. Many of which are described to express autophagy-mediated mechanisms as the first phase and apoptosis-like mechanisms in the last

phase of cell death, projecting a transition from autophagy to apoptosis (Das et al., 2021a). A cysteine cathepsin inhibitor 13b in *L. major*, Yangambin in *L. amazonensis*, cryptolepine in *L. donovani*, miltefosine in *L. major*, and C5 (β-carboline compound) in *L. amazonensis* have appeared to display both autophagy and apoptosis (Basmaciyan et al., 2018; Das et al., 2021b; Mendes et al., 2016; Neto et al., 2011; Scariot et al., 2017; Schurigt et al., 2010; Sengupta et al., 2011; Uzcátegui et al., 2007). Other compounds like 17-AAG (a Hsp90 inhibitor), cryptolepine (a natural indoloquinoline alkaloid), and AMQ-j (a quinoline derivative) have exhibited autophagy-related and apoptosis-like processes in *Leishmania spp.*(Antinarelli et al., 2018; Calixto et al., 2018; Campos-Salinas et al., 2013; Petersen et al., 2012; Santos et al., 2014).

The synergy observed between Veratramine and Miltefosine likely arises from the compounds' targeting complementary pathways, while Miltefosine unresponsiveness is frequently linked to impaired uptake, increased efflux, and suppressed apoptosis. Veratramine can partially bypass or override these unresponsive mechanisms by promoting autophagy-related cell death. In combination, lethal stress from both drugs may drive the parasite beyond its adaptive capacity, resulting in greater efficacy at lower dosages and reduced unresponsiveness. This study provides evidence for the use of Veratramine alone and in combination with Miltefosine against Miltefosine-unresponsive *Leishmania donovani*. The observed synergy is likely attributed to mechanisms involving autophagy, offering a promising pharmacological strategy to manage unresponsiveness in VL. Similar to this study, a thiosemicarbazone molecule-miltefosine combination has been reported to induce a synergistic anti-leishmanial effect, leading to excessive autophagy and apoptotic cell death of the parasite, with the least cytotoxic effect on mammalian cells (Guhe et al., 2023; Scariot et al., 2017). Further investigation into dosing regimens, host-cell effects, and *in-vivo* efficacy is imperative.