Studies on Anti-Leishmanial Compound Discovery

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Conclusions

Leishmaniasis is a parasitic disease widely spread in the tropics and subtropics areas of Asia, Africa, America and Europe. It is classified as a Neglected Tropical Disease (NTD) by the WHO. Among the global population worldwide, the *Leishmania*-infected population is approximately 10-12 million, and approximately 0.9-1.6 million new infected individuals are reported annually. Leishmaniasis is said to cause 20-50 thousand deaths annually. Country-wise distribution of the disease shows that India, Nepal, Bangladesh and Sudan contribute 90% of the total cases. In India, the dominant states affected by this disease are Uttar Pradesh, Bihar, Delhi, Jharkhand, West Bengal, Gujarat, Madhya Pradesh, and Kerala. Bihar contributes 40-50% of world leishmaniasis cases and 90% of total cases in India (Efstathiou and Smirlis, 2021; Samuel Singh, 2019). The main factor contributing to the vast spread of the disease is the diversity of the *Leishmania* species.

For decades, only the selected chemicals were administered to people affected by leishmaniasis: antimonials, amphotericin, paromomycin, and miltefosine. The existing chemotherapy is not satisfactory, and the combination therapy recently adapted to treat leishmaniasis is observed to be effective but causes relapse within a short period of administration. Hence, there is a continuous demand for new and efficient drug development for leishmaniasis, which requires more diverse research (Singh et al., 2014). Drug repurposing is a widely accepted and practical approach for neglected diseases as it requires the least time and resources to reach the affected people and makes the treatment economical. The strategy discussed in the present study increases the implementation of existing drugs for a different target, eliminating the initial steps of drug development and making the process rapidly advance towards execution (Pushpakom et al., 2018). A recent literature review suggests new

strategies for treating leishmaniasis, including immunomodulators, nanotechnology-based drug delivery systems, and drug repurposing however these approaches are still under pre-clinical evaluation (Baranwal et al., 2018; Roatt et al., 2020).

Natural metabolites and their derivatives are a well-known source of biologically active compounds effective against many diseases, including leishmaniasis. Various natural compounds belonging to different families of chemicals have been reported to date and provide us with an opportunity to find a drug of interest. This primarily includes alkaloids, lignoids, saponins, lactones, quinones, and flavonoids (Cortes et al., 2020). Studies have shown that flavonoids and alkaloids are widely evaluated for their anti-parasitic properties. Triterpenoids, a subclass of alkaloids, have induced apoptosis and autophagy in *Leishmania sp* (Moulisha et al., 2010). Under the class of terpenes, sesquiterpenes have also shown anti-leishmanial action by depolarising the mitochondrial membrane and causing cell cycle arrest in the G0/G1 phase, leading to cell death by apoptosis (Cortes et al., 2020; Polonio and Efferth, 2008; Tiwari et al., 2017). Two flavonoids, luteolin and quercetin, were extensively evaluated and appear to act on the parasite by (i) cell cycle arrest in the G0/G1 phase leading to cell death by apoptosis and (ii) topoisomerase I inhibition. Patil *et al*. indicated that saponins possess anti-leishmanial activity, α-hederin, β-hederin, and hederagenin were effective against promastigote and amastigote forms. Being amphipathic, saponins act as a surfactant, hence it is anticipated to work on the parasite membrane, leading to a decrease in membrane potential (Polonio and Efferth, 2008; Salem and Werbovetz, 2006). Another natural compound, plumbagin, is a naphthoquinone which leads to topoisomerase II-mediated DNA cleavage and inhibits parasite proliferation (Polonio and Efferth, 2008).

The *in-silico* techniques were adapted to screen the natural product database. Docking and energy analysis have led us to the natural products, namely, Veratramine, Azulene, Hupehenine and Hederagenin, as the most potent compounds for possible inhibition of the LdDPCK

enzyme. Three of these compounds are classified as alkaloids. Veratramine, an alkaloid, is isolated from rhizomes of *Veratrum* and is reported to be used to treat tumours by inhibiting its signal transduction (Wang et al., 2008). Hupehenine is an isosteroidal alkaloid isolated mostly from *Bulbus fritillariae*, which is reported to possess antitussive effects (Wu et al., 2018). A pentacyclic triterpenoid saponin, hederagenin, is extracted from the *Hedera helix* and has numerous therapeutic effects, such as anti-inflammation, anti-depressant, liver protection, anti-aggregation of platelets, and hyperlipidemia therapy (Lu et al., 2015). Hederagenin has been reported to have an antileishmanial effect on *Leishmania donovani* promastigotes with an IC_{50} of 9.12 ± 0.29 µg/ml (Nkwenti Wonkam et al., 2020). Majester-Savornin *et al*. also indicated the inhibitory effect of hederagenin on *L. infantum* and *L. tropica* (Majester-Savornin et al., 1991), while an IC_{50} of 61.6 ± 0.25 µM has been recorded for intracellular *L. infantum* amastigotes (Rodríguez-Hernández et al., 2016). Azulenes are an isomer of naphthalene, also chemically known as cyclopentacycloheptene and are distilled from camomile oil (Leino et al., 2018). Azulene has appeared to treat HIV (Peet et al., 2016) and is also reported to have antileishmanial activity against *L. amazonensis* (Keshav et al., 2021).

As indicated, it is evident that these natural products, viz. Veratramine, Azulene, Hupehenine and Hederagenin have countless therapeutic values. Additionally, the cell proliferation test by using the MTT assay revealed a significant decrease in the viable cells corresponding to increasing concentrations of the tested compounds. Hupehenine (IC_{50} = 7.34 ± 0.37 µM) showed the best inhibition, followed by Veratramine (IC_{50} = 12.46 ± 2.28 µM). It was observed that Azulene (IC_{50} = 24.42 ± 3.28 µM) and Hederagenin (IC_{50} = 23.36 ± 0.54 µM) showed similar inhibition on promastigotes. However, the classical dose-dependent response may not be manifested by the tested compounds, as the IC_{50} values were calculated with an asymptote at 0% acquired by treatment with a miltefosine concentration at which complete inhibition of the proliferation of promastigotes was observed. The CC_{50} values for the J774A.1 cell line was

observed to be the best for Hupehenine (CC_{50} = 33.28 ± 2.39 µM) and Veratramine (CC_{50} = 21.91 ± 0.14 µM). And also, the SI values of Hupehenine (SI = 4.5) and Veratramine (SI = 1.7) were comparatively higher than Azulene (CC_{50} = 26.42 ± 0.0 µM and SI = 1.0) and Hederagenin (CC_{50} = 24.21 ± 0.3 µM and SI = 1.0). This indicates that Azulene and Hederagenin are equally toxic to J774A.1 cells and promastigotes. Although the individual effect of Azulene and Hederagenin on J774A.1 macrophage is not acceptable, but a safer and much lower concentration of these compounds can be used in synergy with other compounds to give the best results to inhibit *Leishmania donovani*. The *in-silico* studies were effectively executed and also validated the system in *in-vitro* models. The data deducted from the study have to be further validated by *in-vivo analysis, as the effect of these compounds could differ in the in-vivo* model from the parasites and mammalian cell line cultured in media.

Patients afflicted with VL typically receive treatment involving the administration of a polyene antibiotic, amphotericin B, and an alkyl-phospholipid, miltefosine (Kayser et al., 2002). Both amphotericin B and miltefosine have been documented to induce apoptosis-like cell death in *Leishmania donovani* (Mendes et al., 2022; Paris et al., 2004). Apoptosis, a genetically regulated form of programmed cell death initially believed to be exclusive to multicellular eukaryotes, has been identified in unicellular eukaryotes, including various species of *Leishmania*, such as *Leishmania donovani* (Bera et al., 2003; Lyu et al., 2015). Classical indicators of apoptosis in *Leishmania* encompass cell shrinkage, nuclear condensation, DNA fragmentation, loss of mitochondrial membrane potential, release of mitochondrial cytochrome c, increased reactive oxygen species, and release of caspase-like proteases (Basmaciyan & Casanova, 2019; Neto et al., 2011; Paris et al., 2004). Autophagy, a conserved mechanism in eukaryotes crucial for maintaining cellular homeostasis and coping with cellular stress, involves the degradation of cellular contents such as organelles, proteins, and macromolecules in lysosomes, followed by recycling for biomolecule synthesis (Bera et al., 2003; Lyu et al.,

2015). The interplay between autophagy and cell survival is intricate and contingent upon various factors. While autophagy can serve as a pro-survival mechanism under certain circumstances by eliminating damaged cellular components and promoting cell viability, excessive or prolonged autophagy in stressful conditions may lead to cell death, known as autophagy-mediated cell death or type II programmed cell death (Lyu et al., 2015).

In the investigation, treatment with IC_{50} Hupehenine and IC_{50} Veratramine led to a ROS increase of no more than 20%. The cell cycle arrest was detected at the G1 phase in *L. donovani* promastigotes following exposure to Veratramine and Hupehenine, evidenced by an elevated G1 population. This suggests that these drugs impede growth and proliferation, which is further supported by morphological alterations like cell swelling, distortion, flagellar abnormalities, and flagellar loss observed through SEM. To explore whether cell death is mediated by apoptosis, phosphatidylserine externalisation and mitochondrial membrane potential (MMP) were examined. While the number of FITC-positive cells increased with rising drug concentrations, they did not exceed 30%. The change in MMP also did not conform to the classical apoptosis pattern. Specifically, higher drug concentrations did not consistently lead to decreased MMP and subsequent cellular respiration decline, a hallmark of apoptosis. Interestingly, at higher drug concentrations ($2 \times IC_{50}$), there was no significant MMP loss, contrary to lower concentrations (IC_{50}). This suggests that molecular mechanisms promoting increased autophagy may counteract cellular distress collectively, potentially allowing *L. donovani* mitochondria to restore MMP at higher drug concentrations (Haeussler et al., 2020).

Gene expression levels do not always align with protein expression levels due to regulatory processes post-to-mRNA synthesis, involving post-transcriptional and post-translational modifications. For instance, a low translation rate can yield highly stable proteins, resulting in high cellular concentrations, and vice versa. This discrepancy could explain the findings

regarding the ATG8A gene (Greenbaum et al., 2003; Karamysheva et al., 2020). However, increased expression of ATG7 and ATG8B genes corresponds with enhanced autophagic vacuole staining using CYTO-ID dye after treatment with IC_{50} concentrations of both Hupehenine and Veratramine (Giri & Shaha, 2019; Liu et al., 2023; Singh et al., 2017). Thus, while the drugs induce cell death in *L. donovani*, it appears to be a form of programmed cell death distinct from apoptosis (type I programmed cell death). The MMP data pattern suggests a cellular attempt to withstand increased or extreme stress, potentially indicating autophagy, a pro-survival mechanism. Prolonged stress-induced autophagy is characterised by extensive cytoplasmic vacuolization, eventually leading to self-destruction and cell death. This is further supported by increased acidic organelle and autophagic vacuole formation in *L. donovani* promastigotes at 2 × IC_{50} drug concentrations compared to IC_{50} drug concentrations (Neto et al., 2011; Thomé et al., 2016). Cell cycle arrest, morphological abnormalities, and autophagic organelle production validate the direct association of cell death with autophagy.

Evidence has shown Veratramine toxicity in human GBM cell lines, primarily through G0/G1 phase cell cycle arrest and inhibition of the PI3K/Akt/mTOR signalling pathway (Kim et al., 2022; Seale & McDougal, 2022). Additionally, it has been found to hinder prostate cancer proliferation via the ATM/ATR and Akt pathways. Veratramine also exhibits hypotensive and analgesic effects, surpassing the efficacy of common analgesic drugs like pethidine (Li et al., 2019; L. Wang et al., 2008). Yin *et al.* revealed Veratramine's ability to impede liver cancer proliferation through autophagy-mediated apoptosis by suppressing the PI3K/Akt/mTOR signalling pathway (Yin et al., 2020b). Traditionally, Hupehenine is used in Chinese medicine to alleviate colds and congestion. It also shows promising therapeutic effects against neurodegenerative diseases (Ghanem et al., 2021). Pharmacokinetic studies report a bioavailability of 13.4% for Hupehenine (Wen et al., 2015). In addition to the steroidal

alkaloids discussed here, antimicrobial peptides have been identified as agents capable of activating autophagy-mediated cell death in *Leishmania donovani* (Bera et al., 2003).

In summary, Veratramine and Hupehenine effectively hinder the growth and proliferation of *L. donovani* promastigotes. The flow-cytometric and microscopic examinations suggest that this inhibitory effect does not stem from apoptosis-like cell death; rather, the analysis indicates it arises from autophagy-mediated cell death. Further validation of the role of these steroidal alkaloids in autophagy-mediated cell death in *Leishmania donovani* is warranted, necessitating time-dependent proteomic studies employing multiple markers to corroborate the current findings. Nevertheless, Veratramine and Hupehenine hold promise for exploration against other closely related parasitic diseases such as sleeping sickness and chagas disease.

Further, this study also 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 ($28.81 \pm 0.20\ \mu M$) against miltefosine-unresponsive *L. donovani* is over twice that required for the AG83 (sensitive) strain ($12\ \mu M$), 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.

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 indicates 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, cell rounding, flagellar deformation, and cellular distortion across all treatments, 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 might not involve apoptotic mechanisms, indicating the toxic effect induced by other mechanisms. Strangely, Veratramine was 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 a protective mechanism (autophagy) in *Leishmania* previously noted. The level of acidic organelle production in combination therapy may reflect the interplay between drug actions, leading to cell death. CYTO-ID staining revealed that both Veratramine and the Miltefosine-Veratramine combination 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 several compounds that have been 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 positive characteristics (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.