General Abstract

About 1 billion individuals are affected by the Neglected Tropical Diseases (NTDs) worldwide. Leishmaniasis, a Neglected Tropical Disease often referred to as 'kala-azar', poses a significant global health threat. There are three main forms of Leishmaniasis: cutaneous, mucocutaneous, and visceral leishmaniasis (VL), with VL being the most concerning, as it could be fatal if not treated sooner. Around 90,000 new global cases of VL are reported annually by the WHO. While there are drugs for treating the disease, their effectiveness is hampered by their toxicity and the development of drug resistance in parasites. Therefore, more efficient drug discovery and vaccine development are the need of the moment. Coenzyme A (CoA) is a signature molecule that is required in various metabolic pathways for cell survival and proliferation. CoA is a cofactor that is involved in energy generation, protein modifications, differentiation, membrane trafficking, and other fatty acid metabolisms. Inhibition of CoA can be an excellent target to treat VL.

In this research, our focus was on identifying inhibitors targeting Dephosphocoenzyme A kinase (DPCK), with the aim of disrupting Coenzyme A synthesis and ultimately eradicating the parasite *Leishmania donovani*. Dephospho coenzyme A kinase (DPCK), the last enzyme in

Coenzyme A synthesis, was identified as a promising target for developing novel therapeutics against *Leishmania donovani*. Using molecular docking and simulations, Veratramine and Hupehenine were screened from a natural products database and demonstrated strong binding affinities for DPCK. These in-silico findings were further verified by *in-vitro* assays, which revealed that the IC_{50} of Hupehenine and Veratramine were 7.34 ± 0.37 μM and 12.46 ± 2.28 μM, respectively.

Flow cytometry and fluorescence imaging techniques were employed to elucidate the mode of cell death induced by these compounds. Inhibition of CoA biosynthesis in *Leishmania* would lead to modification of several essential metabolic pathways, resulting in reduced ATP production, alteration in the TCA cycle, protein modifications, altered membrane transportation, accumulation of metabolic intermediates and their byproducts, and so on. These changes would trigger cell survival pathways, mainly inducing autophagy, and the prolonged stress leading to excessive autophagy might follow cellular death. This mechanism is supported by the flow cytometric data depicting no signs of apoptosis, such as phosphatidylserine externalisation, ROS generation or the loss of mitochondrial membrane potential. However, exhibiting obstructive morphological changes and cell cycle arrest at the G0/G1 phase, resulting in promastigote inhibition and eventually cell death. The increased acidic vacuole production confirmed by AO staining and autophagic vacuoles evaluated by CYTO-ID staining demonstrated that the promastigotes' inhibition and death are autophagy-mediated. Unlike most drugs, which show typical cell death by apoptosis, evidence indicates cell death involving autophagy, which was further assisted using ATG gene expression by RT-qPCR. This study shows the potential of Veratramine and Hupehenine as effective anti-leishmanial agents and features the importance of DPCK as a drug target against Visceral leishmaniasis.

For over two decades, miltefosine has been the only available oral drug to treat VL worldwide. The emergence of miltefosine resistance in *Leishmania donovani* has turned into a critical

challenge for the treatment of VL. The inhibitory effect of veratramine on miltefosine-unresponsive *Leishmania donovani* has been evaluated, as veratramine has proved to be effective on the miltefosine-sensitive strain. Veratramine demonstrated an IC_{50} of 28.81±0.20 µM, compared with 12 µM in sensitive strain, indicating partial resistance. 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, demonstrating good synergism. 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 promastigotes after the treatment. The requirement of lower concentrations of the drug compounds in combination is of particular interest in the case of miltefosine-unresponsive *Leishmania donovani* strain, as it would help overcome the resistance, since the combination therapy is more efficient and less toxic. The inhibitory effect of the miltefosine and veratramine monotherapy and the miltefosine-veratramine combination therapy was also observed through morphological changes analysed by SEM. Cell rounding, cell swelling, flagellar loss, flagellar abnormalities and other morphological deformations were observed. Miltefosine is known to act on *Leishmania* by inhibiting lipid metabolism, increasing ROS, disrupting mitochondrial membrane potential and leading to the induction of apoptotic cell death. The miltefosine resistance reported in *Leishmania* is due to some genetic modification which leads to downregulation of protein transporters like miltefosine transporter (LdMT) and its beta subunit (LdROS3), which leads to reduced drug uptake into the cells. Also, there is efflux of miltefosine due to the overexpression of P-glycoprotein, which is one of the ABC transporters which utilises ATP to actively pump miltefosine out of the cell, reducing its cellular concentration. There is also an alteration in lipid metabolism, which reduces miltefosine effectiveness, and improved oxidative stress management, due to the expression of certain genes that modify oxidative metabolism.

From the previous experiments, it was observed that veratramine leads to morphological changes, cell cycle arrest, an increase in acidic organelle production, an increase in autophagic gene expression and induction of autophagic vacuole production, leading to autophagy-mediated cell death in AG83 *Leishmania donovani* strain. In this study, the combination of miltefosine-veratramine combination therapy was evaluated for its mode of action in the miltefosine-unresponsive *Leishmania donovani* strain. The results indicate that the miltefosine-veratramine combination leads to inhibition of cell growth and proliferation similar to the mechanism that was observed in the miltefosine-sensitive strain. It could be explained by the evidence that the veratramine inhibits the CoA synthesis pathways, which leads to reduced energy production, alteration of protein modification and lipid composition. This would have improved miltefosine uptake into cells by altering miltefosine transporters and reducing efflux from cells through reducing the activity of ABC transporters by depleting the available ATP, which eventually increased cellular miltefosine concentration. Although veratramine could have caused some chemical changes in the promastigote cells, it is less likely that it could alter the *Leishmania* genetics; therefore, the other supporting mechanisms of miltefosine resistance, including improved oxidative stress regulation and apoptosis-inhibition, might not be affected. The increased cellular miltefosine concentration might have complemented the effect of veratramine, which leads to the induction of increased autophagic stress as observed through AO and CYTO-ID staining, leading to *Leishmania donovani* inhibition and autophagy-mediated cell death. The current study offers a promising pharmacological strategy to manage resistance in VL, and further research, including *in-vivo* studies and other pharmacological analyses, is essential.