

## Chapter 6

### Summary and Future aspects

The leading cause of global morbidity and mortality is an infectious disease, which is known to pose serious health threats to the world's populations dating back to ancient Egypt. In the 14<sup>th</sup> Century alone, infectious diseases were responsible for decimating 20-45% of the world's population. Among infectious diseases, many of them fall under the category of neglected diseases. Leishmaniasis is one of the neglected tropical diseases caused by different species of leishmania. According to WHO, there are 350 million people at risk of infection, and, 700,000 to 1 million new cases and 25,000 to 26,000 deaths occur annually worldwide. This disease was prevalent in tropical and subtropical regions, notably Latin America, Southeast Asia, East Africa, and North Africa. However, shifts in climatic patterns, co-infections with other diseases like HIV, and population mobility have altered its distribution pattern and heightened its susceptibility. It is also called a 'poverty-associated disease' as it is prevalent among economically weakened populations who have limited healthcare facilities and are unable to influence on governmental authorities. Currently, there is no vaccine available against this disease; the whole treatment depends on chemotherapeutic options. These options also have several drawbacks, like toxicity, high cost, challenging route of administration, and parasite resistance. Additionally, the pharmaceutical industry pays minimal attention to this disease due to its limited commercial returns, which inhibits research and the development of innovative therapeutics. In light of these challenges, the quest for novel therapeutic targets becomes imperative. The discovery of novel drug targets and repurposing of existing drugs with novel targets is pivotal for advancing new drug development efforts. Searching for novel target necessities focuses on the leishmania life cycle and its metabolic pathways.

Amastigote resides in the phagolysosomes, which have a low pH (~ 5.4), high temperature (35 °C to 37 °C), and hydrolases and NADH oxidase enzymes, which produce an anti-microbial oxidative burst. The studies indicate that lesion amastigotes, to protect themselves from these situations, enter into a semi-quiescent state and show a stringent response to evade the activation of host cell microbiocidal processes. The <sup>13</sup>C-tracer studies have demonstrated that lesion amastigotes, like promastigotes, preferentially utilize sugars but at lower rates. It utilizes fatty acids as a significant carbon source and relies on the  $\beta$ -oxidation of fatty acid as it produces acetyl-CoA, mostly employed in the TCA cycle. This cycle provides glutamate, glutamine, and aspartate that help in the synthesis of the nucleotide, thiol, and amino sugar, which help amastigotes in their growth, survival, and defense from host-microbiocidal processes.

Here, we have focused on Citrate synthase, the first TCA cycle enzyme involved in the TCA cycle continuation and ATP production in mitochondria and fatty acid & cholesterol synthesis in the cytosol. It also functions as an inflammatory signal and plays a crucial role in regulating immune cell metabolism. This enzyme has substantial sequential and structural differences with human citrate synthase.

In this study, we have selected citrate synthase as a drug target based on its functionality and sequential & structural differences with human citrate synthase. The crystal structure of this protein is not available; thus, its model was generated using the ITASSER server, and the best model was selected based on its C and TM scores. The selected model energy was minimized and validated through RAMPAGE and ProSA server based on the Ramachandran plot and Z-score. After validation, we served this model for molecular docking with 1065 natural and 1565 FDA-approved compounds sourced from ZINC 15 databases, aiming to identify potential compounds for LdCS. The top five hits, such as Abemaciclib, Bazedoxifene, Vorapaxar, Imatinib, and Amyral, were selected based on the lower binding energy with LdCS and HsCS,

difference in binding energy with HsCS and also interaction with the active site residues of the LdCS protein. Notably, all selected molecules adhered to Lipinski's rule with zero violations, which assesses the oral activity of drugs. MD Simulation investigation was carried out with these ligands based on RMSD, RMSF, Radius of gyration, and Hydrogen bond analysis.

The protein-ligand complexes' structural stability, lack of fluctuation in their catalytic area, and compactness demonstrated that these ligands would interact with proteins in a stable and robust manner at their binding sites. All ligands had formed hydrogen bonds which demonstrate its complexity and rigidity. MM-PBSA investigations were conducted after the MD Simulation to examine the variation in their binding energy. All complexes had shown lower binding energy and majorly a hydrophobic contact. Pre-residue energy decomposition studies were also carried out and found that a few residues like Try 238, His 242, His 245, Asp 264, Phe 269, Leu 273, and Asn 285 were commonly involved in interaction with the active site of LdCS. The pharmacokinetics studies were performed based on ADMET analysis, as most drug development failures occur due to inadequate pharmacokinetics. All selected ligands exhibited good water solubility, strong CaCO-2 permeability, good intestinal absorption, and good skin permeability without skin sensitivity issues. They also demonstrated a more consistent medication distribution in blood plasma and a lower unbound fraction level. Altogether, these ligands showed good absorption, solubility, permeability, adequate metabolism, and negligible toxicity.

Further, biophysical and biochemical characterization studies were performed to understand the structural and functional properties of the LdCS protein. As we all know, the leishmanial parasite has a digenetic lifecycle encompassing two forms, promastigote and amastigote, that exhibit fluctuations in pH and temperature. This parasite might have alternation in its protein structure to maintain itself in a changing environment. The Ldcs gene was cloned and expressed in the bacterial expression system; thus, there is a chance of structural alteration and functional

ambiguities. The LdCS protein was purified through affinity chromatography, and its purity and size were confirmed through SDS-PAGE electrophoresis. The far-UV and fluorescence spectroscopy were employed to elucidate the protein's secondary and tertiary structures. Circular dichroism spectroscopy provides insights into protein secondary structure and unordered conformations based on their magnitude and wavelengths of ellipticity bands. The secondary structure of LdCS revealed predominantly alpha-helical content and elevated ellipticity at physiological pH, indicating highly folded protein. However, at high and low pH, its ellipticity gets reduced, which might indicate a loss of secondary structure. Thermal denaturation studies were also performed with this protein, demonstrating its resistance to structural changes upon unfolding. However, once denatured, unable to revert to its original structure. The melting temperature was found to be 44 °C.

The protein contains fluorogenic amino acid residues, specifically indole ring containing tryptophan, which lead to large variation in their fluorescence properties upon modification. This principle was employed to evaluate the conformational changes of the tertiary structure of protein. It is also well known that the emission maxima of water-accessible tryptophan residues occur above 350 nm, whereas the completely buried tryptophan residue in a hydrophobic environment will be near 330nm. The emission maxima of LdCS in intrinsic fluorescence spectroscopy were found near buried tryptophan residues without any major shift upon pH change, implying that the LdCS protein was present in its native folded state and retained its conformational stability. However, in extrinsic fluorescence spectroscopy, there was an increase in fluorescence intensity with large red shift observed at acidic pH, meaning that the protein was exposed to a hydrophobic environment and adopted a partially and fully unfolded state. Further, to reveal the structural features of the LdCS protein, the intrinsic fluorescence-based quenching studies were performed using potassium iodide and acrylamide quencher. Compared to potassium iodide, the decrease in fluorescence intensity and higher

quenching constant in the presence of acrylamide revealed that most of the tryptophan residues are hidden inside the core of the protein and accessible to the acrylamide. The urea and GnHCl-mediated unfolding studies of LdCS were performed using intrinsic fluorescence spectroscopy, and their data were normalized and fitted into a two-state equation. The unfolding transition concentration of urea was found to be 2.85 M and for GnHCl 1.85 M. To assess the potential of LdCS as a drug target and to evaluate the binding affinities of selected compounds such as Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib through *in-silico* studies, its intrinsic fluorescence spectrum was observed through intrinsic fluorescence spectroscopy. All selected compounds fluorescence intensities get decreased in a concentration-dependent manner upon binding of LdCS, and also, its binding affinities were observed to be greater than  $10^{-4}$  M, which means these compounds had shown stronger binding affinities.

The functional characterization studies of LdCS were performed by evaluating enzyme activities based on yellow TNB complex formation at 412 nm. The impact of pH on catalytic activities was evaluated in the range of 4 to 10 to choose the best assay conditions for kinetic characterization and inhibition studies since the medium pH affects the protonation state of amino acids linked to catalysis and/or intermolecular interaction. The optimum pH was found to be 7.0. Kinetic characterization studies with both substrates, i.e., acetyl CoA and OAA, were conducted to screen inhibitors and determine their modalities. The  $K_m$  of acetyl CoA was larger than OAA, suggesting a lower affinity for acetyl CoA. Inhibition studies were formed with selected compounds to evaluate their mode of inhibition and inhibition constant. All selected compounds exhibited a competitive mode of inhibition towards acetyl-CoA and an uncompetitive mode of inhibition towards OAA. Their inhibition constant values were found in the range of 2 to 3  $\mu$ M. Further, antileishmanial and cytotoxicity studies were performed with selected compounds using promastigote and amastigote forms of leishmania and murine macrophages J774 A 1. These compounds decreased the cell viability of promastigote and

infectivity of intra-macrophagic amastigote in dose-dependent manners, and the IC<sub>50</sub> values in case of promastigote were found to be  $0.92 \pm 0.02 \mu\text{M}$ ,  $0.65 \pm 0.09 \mu\text{M}$ ,  $6.1 \pm 0.91 \mu\text{M}$ , and  $23.9 \pm 1.27 \mu\text{M}$  & the EC<sub>50</sub> values in case of intra-macrophagic amastigote were found to be  $1.52 \pm 0.37 \mu\text{M}$ ,  $2.11 \pm 0.38 \mu\text{M}$ , and  $10.4 \pm 1.27 \mu\text{M}$ , respectively for Abemaciclib, Bazedoxifene, Vorapaxar and Imatinib. These values were lower than Miltefosine as their IC<sub>50</sub> 13.6 and EC<sub>50</sub>  $13.4 \pm 0.53 \mu\text{M}$  were found. Vorapaxar showed no cytotoxic effects on macrophage viability up to 500  $\mu\text{M}$  whereas other compounds Abemaciclib, Imatinib, and Bazedoxifene exhibited less cytotoxicity towards macrophages with CC<sub>50</sub> values  $83.35 \pm 0.77 \mu\text{M}$ ,  $43.7 \pm 0.42 \mu\text{M}$ , and  $11.8 \pm 0.56 \mu\text{M}$ , respectively. The selectivity index of Abemaciclib, Bazedoxifene, and Vorapaxar in the case of both promastigote and Intra-macrophagic amastigote was found to be superior to Miltefosine.

To evaluate its mechanism of action and mode of death, the anticancer drug Abemaciclib was selected based on its lower binding energy, a significant difference in binding energy with HsCS and interaction with the active site of LdCS in *in-silico* studies, and also their selectively inhibitory effect against both promastigote and Intra-macrophagic amastigote at micromolar concentration without much toxicity towards macrophages. The Abemaciclib-treated promastigotes showed a typical alteration in morphological patterns such as rounding, cytoplasmic shrinkage, and loss of flagella under scanning electron microscopy compared to cylindrical shape and long flagella containing normal promastigote cells. The structure of Mitochondria was analyzed in treated cells using MitoSoX red, which showed the distortion of well-defined mitochondrion and accumulation of dye in the cytoplasm with bright red aggregation. The shift of fluorescence intensities and the production of 33 % to 50% ROS positive cells were observed in Abemaciclib treated cells. High levels of ROS production can result in mitochondrial malfunction, which is further evidenced by the striking drop in the JC-1 dye's red/green fluorescence intensity. The damaged mitochondrial membrane might activate

the chain of caspase-like proteases, which lead to the production of endonucleases and might be the reason for the fragmentation of genomic DNA. In Abemaciclib-treated cells, fragmentation of genomic DNA was observed in DNA laddering assay, and elevation of Sub-G1 cell population up to 44% was observed in cell cycle assay, which indicates the apoptotic mode of death mechanism. It was further confirmed through double staining with Annexin V and PI. Annexin V has an affinity for phosphatidylserine, which occurs in apoptotic cells, whereas PI has an affinity for necrotic cells. Normal cells do not have an affinity for both dyes. There were, 64% of cells observed in the apoptotic state in treated cells.

Altogether, the LdCS protein, after expression in a bacterial system, occurs in correctly folded form and maintains its conformational stability and catalytic activity, ensuring its functionality and effectiveness. The selected compounds showed significant differences in binding energy with HsCS, stability, less fluctuation in the catalytic site, and high compactness in *in-silico* studies, demonstrating their effectiveness and safety. Further, these compounds showed good binding energy in fluorescence studies and a competitive mode of inhibition in *in-vitro* studies, reducing the likelihood of off-target binding. These compounds selectivity in antileishmanial activities over toxicity compared to Miltefosine and also the accumulation of ROS, depolarization of the mitochondrial membrane, DNA degradation, and cell cycle arrest confirm the action of Abemaciclib on mitochondria and apoptotic mode of death, which drive its progression towards drug development pipelines. In the future, we can do *in vivo* research and clinical trials based on these findings. We can then submit for supplemental new drug applications and proceed with marketing and commercialization.