Understanding pyrimidine and aspartate metabolism in the growth and infectivity of *L. donovani*

A Thesis
Submitted in Partial
Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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July 2017

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Dedicated to
My ever amazing parents,
inspiring mentors
and curious researchers

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

STATEMENT

I hereby declare that the matter embodied in this thesis titled **"Understanding pyrimidine and aspartate metabolism in the growth and infectivity of *L. donovani*"** is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India under the supervision of **Prof. Vikash Kumar Dubey**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred. Further the data in the thesis are collected by me. I certify that there is no fabrication or manipulation of data in the thesis.

Date: July, 2017

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DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

CERTIFICATE

It is certified that the work described in this thesis **"Understanding pyrimidine and aspartate metabolism in the growth and infectivity of *L. donovani*"** by **Mr. Kartikeya Tiwari** (Roll No. 11610612), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under my supervision at the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

Prof. Vikash Kumar Dubey

(Thesis Supervisor)

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Whatever good have been done and whatever good is too be done from my side will always be a reflection of my parents and peers. As always this work is also not free from errors. I hope peers will forgive me for this. This is what I was able to do with my limited capacities. Finally I seek blessings from my parents and peers and dedicate this work to them.

Kartikeya Tiwari
July, 2017

Abbreviations

AnsA	:	L-cytoplasmic asparaginase (Type I)
AnsB	:	L-asparaginase like protein (Type II)
BLAST	:	Basic Local Alignment Search Tool
BSA	:	Bovine Serum Albumin
CL	:	Cutaneous Leishmaniasis
CA-asp	:	N-Carbamoyl-L-Aspartate
DHO	:	Dihydroorotate
DHOase	:	Dihydroorotase
DNA	:	Deoxyribonucleic Acid
EDTA	:	Ethylene Diamine Tetra Acetic Acid
FACS	:	Fluorescence Activated Cell Sorting
FBS	:	Fetal Bovine Serum
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
*Ld*DHOase	:	Dihydroorotase of *Leishmania donovani*
MCL	:	Mucocutaneous Leishmaniasis
mRNA	:	Messenger Ribonucleic Acid
MTT	:	[(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)]
NaCl	:	Sodium chloride
NCBI	:	National Center for Biotechnology Information
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase Chain Reaction
PI	:	Propidium iodide
PKDL	:	Post Kala Azar Dermal Leishmaniasis
RNA	:	Ribonucleic Acid
SDS	:	Sodium Dodecyl Sulphate
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

List of Figures

List of Tables

Contents

CHAPTER 1

Biochemistry of *Leishmania* in the context of pyrimidine and aspartate metabolism*

ABSTRACT

Visceral Leishmaniasis (commonly known as *Kala azar*) is caused by a unicellular parasite *Leishmania donovani*. It continues its killing spree amounting to significant mortalities each year mainly due to lack of effective treatment regimens and the prominence of the disease in the poor. Emerging drug resistance especially in the Indian subcontinent is posing a serious threat in the eradication of this disease. An understanding of biology of the parasite will help in the management and elimination of this fatal disease. The parasite in its dimorphic form thrives unchecked in the dual host system outsmarting the immune mechanisms. Immune evasion mechanisms employed by the parasite has receded the development of an effective vaccine for the Visceral Leishmaniasis (VL). Though a plethora of treatment regimens for curing VL are available, they have limitations related to toxicity, high cost and resistance issues. A better understanding of the biological processes and pathways of the parasite will help in taking the gaint leap towards the development of effective treatment and vaccines for VL.

*** Part of the work is submitted for publications**

1.1 Introduction

Leishmania donovani, the protozoan parasite in its digenetic and dimorphic form causes visceral leishmaniasis (VL), the second most neglected tropical disease claiming numerous lives worldwide. A worldwide attempt to curb this disease either by development of efficient drugs or by vaccines is in progress. This work needs urgency as the current treatment regimen suffers from major drawbacks like severe toxicity and resistance issues. Researchers continue to probe various metabolic pathways of this parasite in a search for effective drug targets. A hunt for the soft targets in the protozoan parasite has led to an overwhelming knowledge of the drug targets.

Visceral leishmaniasis (VL) is a neglected tropical disease prevalent in 98 countries across the globe and causing the death of around 50,000 people per year *(Maran et al, 2016)*. VL is caused by an obligate intracellular vector borne parasite *Leishmania donovani*. The deadly parasite maintains its dualistic lifestyle by perpetuating itself in two hosts: Sandfly (*Phlebotomus* in the old world and *Lutzomyia* in the new world) in the form of slender shaped promastigote having flagella and in vertebrates in the form of rounded amastigote devoid of flagella. In the Indian subcontinent the worst affected areas lie in the northeastern parts of India where it is spread by *Phlebotomus argentipes* sandflies (dipteran insects of Psychodidae family) *(Cameron et al, 2016)*. More than 20 species of *Leishmania* causes Leishmaniasis and the transmission of the parasite is brought about by 30 different species of *Phlebotomine* sandfly *(Chappuis et al., 2007)*.

In addition to the existing havoc caused by the parasite in the past the issue of visceral leishmaniasis and HIV coinfection have started to surface in the recent years which has resulted in the poor efficacy of the current treatment options as well effective presentation of the parasite *(Monge-Maillo and López-Vélez, 2016)*. Emerging drug resistance especially in the Indian subcontinent is posing a serious threat in the eradication of this disease. An understanding of biology of the parasite will help in the control and eradication of this deadly disease.

1.2 Leishmaniasis: Historical perspective

Visceral leishmaniasis is referred to as *Kala azar* (because of hyper pigmentation) in the Indian subcontinent. The earliest reports of the occurrence of *kala azar* dates back to 1824

when the British surgeon William Twinning in Calcutta reported the symptoms like splenic enlargement, acute anemia and intermittent fever in the local population. An epidemic broke out in the regions of Assam and Bengal in the year 1858 which was also resistant to quinine and had malaria like symptoms; it also claimed numerous lives during the epidemic. Initially the disease was misconstrued to be a form of malaria. In the forthcoming years the disease started spreading westwards of Bengal and also in Assam. The severity of the disease can be estimated by the fact that in some cases whole villages were wiped out because of the infection. In the year 1898 the famed parasitologist Ronald Ross inspected cases of *kala azar* in Assam and concluded that it was a manifestation of untreated malaria. In 1903 a case was reported by William Leishman in British Medical Journal of an infected soldier in Dum Dum near Calcutta in whose smear some unknown parasites stained with Romonowsky stain were observed. But he suggested that *kala azar* belonged to the category of trypanosomiasis. In the same year Charles Donovan, who worked in the physiology division of Madras Medical College identified a parasite of the same morphology from an autopsy that resembled that of Leishman's sample. Initially the newly discovered parasites were termed as *Piroplasma donovani* by Laveran, but later were referred to a *Leishmania donovani* by Ronald Ross. In 1904 the parasite was also identified from Sudan by Dr. Sheffield Neave. In 1904 *Leishmania* was found in children suffering from infantile splenic anaemia *(WHO Report, 2012)*. *Leishmania* from the mucosal lesions of patients was identified in Brazil in 1912 *(WHO Report, 2012)*. Post kala azar dermal leishmaniasis (PKDL) was described by Brahmachari in India in 1922. *Leishmania donovani* transmission by Phlebotomine sandfly was demonstrated by Swaminath, Shortt and Anderson in India in the 1940s. In the year 1942 sandfly (Phlebotomus) was proved to be a vector of *L. donovani (Gibson, 1983)*.

1.3 Types of Leishmaniasis

Depending on the type of clinical manifestation Leishmaniasis can be divided into 4 broad categories: Cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (also known as espundia), visceral leishmaniasis (VL), and post-kala-azar-dermal-leishmaniasis (PKDL). *Leishmania major* and *L. topica* are the causative agents of cutaneous leishmaniasis which is identified by the presence of skin lesions which are painless and have a self healing tendency. Manifestation is visible after a month or in some cases in a year. Skin lesions emerge out

from papules or nodular plaques. In some cases cutaneous leishmaniasis is followed by mucocutaneous leishmaniasis which leads to the destruction of nasal mucosa and leads to severe disfiguring of skin and lacks self healing properties. In mucocutaneous leishmaniasis the dissemination of the parasites is from skin to nasooropharangeal mucosa. Most cases of mucocutaneous leishmaniasis are caused by *Leishmania braziliensis* and *Leishmania amazonensis*. VL is caused by *Leishmania donovani* (In Indian subcontinent and East Africa), *Leishmania infantum* (In Europe, North Africa and Latin America) and *Leishmania chagasi* (In Mediterranean region, south west and central Asia) and is fatal if left untreated. All age groups are infected by *Leishmania donovani* while infants are infected by *Leishmania infantum*. Incubation period varies from weeks to months but the clinical manifestation is evident after many years. Clinical symptoms are severe in immunocompromised individuals suffering from HIV/AIDS etc. Internal organs like spleen, liver and bone marrow are affected. Visceral leishmaniasis is of two kinds based on the mode of transmission: zoonotic meaning the parasite is transmitted from animal to vector to human while in anthroponotic category the parasite is transmitted from human to vector to human. Humans are rarely infected in the zoonotic transmission. Symptoms like fever, fatigue, weight loss (cachexia: wasting), hepatosplenomegaly (prominence of spleen is more then liver), pancytopenia (anemia, leucopenia, and thrombocytopenia) are evident after an incubation period of 4-6 months. Splenomegaly is often observed in the advanced cases of VL *(Chappuis et al., 2007)*. Lymphadenopathy is common in some regions of Sudan. PKDL (post-kala-azar-dermal-leishmaniasis) is rare in Indian subcontinent and common in Sudan. It arises in complicated forms of visceral leishmaniasis and is characterized by the presence of macula-papular or nodular rashes on face. Poor population of the developing countries like India is mostly affected by visceral leishmaniasis which also accounts for high cost incurred in the treatment of this deadly disease.

1.4 Life cycle of *Leishmania*

1.4.1 Flagellated promastigotes in the sandfly

The parasite has a digenetic and dimorphic life cycle as it maintains itself in two hosts: sandfly and vertebrate host. The parasite is transmitted by *Phlebotomus* (in the old world)

and by *Lutzomyia* (in the new world) genera of the sandfly. It exists in the form of an amastigote (devoid of flagella) in the vertebrate host and as a promastigote (having flagella) in the sandfly **(Figure 1.1)**. When the sandfly takes a blood meal it takes up the amastigote form of the parasite. Female sandflies are poor feeders and they just insert their saw like mouth parts in the skin which creates a wound locally. In a bodily response macrophages infected with amastigotes arrive at the wounded site and are taken up by the sandfly *(Lane, 1993)*. The ingested amastigotes are transferred to the abdomen of the sandfly. In the sandfly abdomen the parasites encounters a different environment in terms of temperature and pH (slightly alkaline). The amastigotes develop flagella at the anterior end and are now called as procyclic promastigotes which are weakly motile. The attachment of promastigotes to the microvilli of abdominal midgut is by means of flagella. Promastigotes divide by binary fission in the sandfly host.The sequence of events that follows in the division is: production of daughter flagellum, division of nucleus and kinetoplast and finally separation by longitudinal fission resulting in two daughter cells. Further the promastigotes differentiate and display glycoconjugates on their surface which help them to migrate to mouth parts, a term known as metacyclogenesis in which procyclic promastigotes differentiate into metacyclic promastigotes. Firstly, these procyclic promastigotes differentiate into the long slender shaped nectomonad stage which is highly motile migratory form. Secondly, nectomonads usually migrate to the anterior midgut and attach to the microvilli lining the midgut epithelium and finally reach the stomadeal valve where they transform into shorter leptomonad promastigotes *(Gossage et al., 2003)*. These leptomonads secrete a gel known as promastigote secretory gel (PSG) which helps in their transmission *(Rogers et al., 2002)*. PSG mainly contains high molecular weight glycoprotein called filamentous proteophosphoglycan (fPPG) *(Ilg et al., 1996)*. This PSG blocks the anterior midgut and forms a plug like structure containing promastigotes which supports the blocked fly hypothesis. A final conversion transforms leptomonads into metacyclic promastigotes which is the mammalian infective stage. The metacyclic promastigotes are highly motile and detach themselves from the midgut epithelium and migrate into the buccal cavity The membrane composition of carbohydrates differs between log phase promastigotes and metacyclic promastigotes, while the later showing a loss of expression of glycolipid which is present in

promastigotes, although lipophosphoglycans present in metacyclic promastigotes aid in their attachment to macrophages *(Killick-Kendrick, 1990)*.

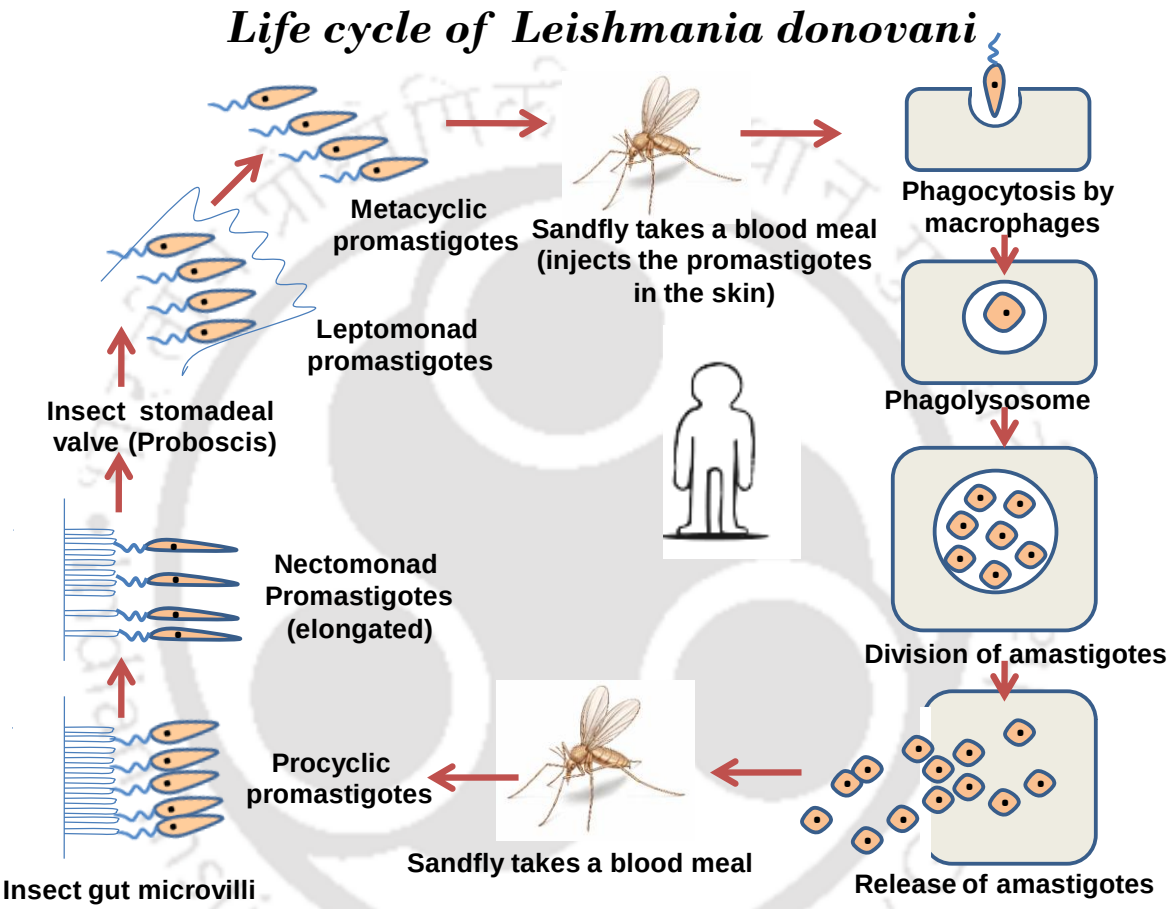


Figure 1.1. Life cycle of *Leishmania donovani*. The parasite maintains its dual existence in sandfly and Human host. The flagellated form of the parasite referred to as promastigote perpetuates in the sandflly until it is transmitted to the host by the sandfly bite. The promastogote looses its flagella in the mammalian host and is referred to as amastigote whose multiplication results in clinical manifestation of the disease.

1.4.2 Amastigotes in the mammalian host

Phagocytosis by macrophages

When the sandfly takes a blood meal it injects the metacyclic promastigotes in the blood stream of an individual and these promastigotes are then immediately phagocytosed by macrophages through the process of receptor mediated endocytosis **(Figure 1.1)**. Whole reticulo-endothelial system is affected through vascular and lymphatic routes followed by infiltration of the bone marrow which results in hepatosplenomegaly and enlarged lymph nodes (lymphadenopathy). Neutrophils followed by natural killer cells (NK cells) are the first cells of the immune system to invade the site of infection by *L. donovani (Muller et al., 2001).* Sandfly saliva contains a peptide called Maxdilan which inhibits the LPS induced TNF-alpha and NOS production by macrophages. Promastigotes interact with bone marrow macrophages (BMMs) through their flagellar tip on the anterior end which is followed by reorientation of the promastigote as it attaches to pseudopods of macrophages from posterior end *(Courret et al., 2002; Forestier et al., 2011).* Interaction of LPG on promastigote surface with TLR2 (Toll like receptor) induces the release of TNF-α and IL-12 from macrophage, however the parasite escapes this response as promastigotes interferes with the release of IL-12 and TNF-α by causing hindrance with the ubiquitination of host protein TRAF6 as they activate host de-ubiquinating enzyme A20 *(Srivastav et al., 2012).* By another mechanism TLR4 induced release of TGFβ causes activation of ubiquitin editing enzyme A20 and Src homology 2 domain phosphotyrosine phosphatase 1 (SHP-1) *(Das et al., 2012).* The host is unable to elicit the humoral immune response as the parasite goes totally unchecked. The first encounter of the parasites in the blood occurs with the host complement system, but the metacyclic promastigotes outsmart the complement mediated lysis by expressing longer chain LPG molecules on their surface which prevents the insertion of the C5-C9 membrane attack complex *(Descoteaux et al., 1999; Puentes et al, 1990).* Phagocytosis is mainly initiated by complement and Fc receptors *(Underhill and Ozinsky, 2002).* The C3b component of complement system is employed by the parasite to adhere to the macrophage surface. Further the phagocytosis in the macrophage is aided by the C reactive protein which interacts with the LPG. The C3b and C3bi interact with the CR1 and CR3 on macrophage surface leading to the entry of metacyclic promastigotes. The parasitic entry through this receptor does not generate reactive oxygen species, which has been proved by many studies

(Wright and Silverstein, 1983) (Ueno and Wilson, 2012). Also this interaction prevents oxidative bursts and IL-12 production by the macrophage which can otherwise eliminate the parasite. Various models of promastigote phagocytosis have been proposed by researchers including conventional zipper vs. coiling coil model *(Rittig et al., 1998).* Why macrophages fail to initiate antimicrobial responses upon parasitic entry? An interesting observation has been put forward in this regard by Laskay in *L. major* in which they showed that the promastigotes are phagocytosed by neutrophils which later become apoptotic bodies which are in turn phagocytosed by macrophages, thus macrophages fail to detect the parasite and it happily survives within the macrophage *(Laskay et al., 2003).* Many other mechanisms of parasitic entry like apoptotic mimicry by displaying phosphatidyl serine (PS) and caveolin based internalization have been defined in many studies *(Wanderley et al., 2006) (Rodriguez et al., 2006).* Other species of *Leishmania* such as *L. amazonensis* can gain entry into the macrophage through other receptors such as heparin sulphate and fibronectin receptor *(Alexander et al., 1999).* The reliance on multiple receptors for gaining entry in the macrophages is an added advantage to the promastigotes as they are spared from destruction. Amastigotes also infect dendritic cells which have been defined as a silent entry mediated by C type lectins, usually dendritic cells are not activated.

Phagolysosome

The phagocytosed parasite is retained inside the phagosome in macrophages. The phagosome (also known as parasitophorous vacuole) fuses with the lysosome resulting in the formation of phagolysosome which has an acidic environment and is rich in hydrolases. The phagosome may fuse with the endosome, but this event is somehow prevented by steric repulsion between their membranes by LPG molecules expressed by promastigotes *(Descoteaux and Turco, 1999).* During the fusion of phagosome to lysosome, for a brief period of time the acidification in the phagosome is prevented by the parasite. It does this in a very interesting way, it inserts the LPG in the lipid microdomains of phagosome membrane which relieves synaptotagmin V off the membrane and this synaptotagmin V is responsible for the recruitement of vescicular proton ATPases (V- ATPases) which leads to the acidification of the phagosome, this allows the differentiation of promastigotes to amastigotes *(Vinet et al., 2009).* Iron also plays a major role in the survival of the parasite as

it is required for its replication and metabolism in the phagosome and thus it uptakes iron in the form of ferrous (Fe^{2+}) and hemin (Fe^{3+}: oxidation product of haem) as it possesses the hemin receptor *(Carvalho et al., 2009).* The host macrophage however pumps iron from phagosome into cytosol through Nramp1, the parasite counteracts this by expressing its own iron transporters, LIT1 and LIT2 *(Kaye and Scott, 2011)*. Survival of the promastigote in the harsh environment of phagolysosome is taken care of by the LPG on the promastigote's surface as well as by the delayed maturation of parasitophorous vacuole. Actin accumulation is a sign of phagosome maturation and this event is accelerated by parasitic protein MSP (major surface protease) which cleaves scaffold protein tyrosine phosphatase (actin remodeler) *(Halle et al., 2009).* RAB7 and LAMP-1 plays an important role in the fusion of endosome and lysosome. In immature DCs the RAB7 recruitement is inhibited by the parasite which ensures long lasting survival in the phagosome. The fusion is also prevented transiently by late expression of LAMP-1 *(Olivier et al., 2005; Scianimanico et al., 1999)*. In the phagolysosome the promastigote differentiates to the amastigote form by rounding up, losing its flagellum and changing its glycoconjugate coat *(Cunningham, 2002)*. During its stay in the phagolysosome various counter intuitive strategies are employed by the amastigote to ensure the survival. The amastigotes are prevented by the action of hydrolases by the anionic LPG on the amastigote surface *(Descoteaux and Turco, 1999).* Cellular signaling involving protein phosphorylation in macrophages (especially tyrosine phosphorylation) is also inhibited by phosphatases of *L. donovani* amastigote and by other unknown strategies *(Kane and Mosser, 2000).* Tyrosine dephosphorylation in proteins has also been reported to be done by activated phosphotyrosine phosphatases (SHP 1) *(Forget et al., 2001).* In a study ERK1 (a MAPK) was found to be dephosphorylated in infected cells *(Martiny et al., 1999)* although contradictory versions have been reported by other groups. Amastigotes divide rapidly in the phagolysosome and finally rupture the macrophages releasing enormous amounts of amastigotes in the bloodstream which further infects more macrophages.

1.5 Evasion of host immune response

Leishmania donovani interferes with the MHC II mediated antigen presention in an ingenious way by enhancing the fluidity of the membrane thus leading to poor accumulation of MHC II molecules in the lipid rich microdomains leading to poor antigen presentation *(Chakraborty et al., 2005)*. Induced nitric oxide synthase (iNOS) in response to IFNγ produced by Th1 cells generates nitric oxide (NO) in macrophages under stimuli such as infection etc. But *Leishmania* infected macrophages fail to produce NO, according to one opinion nitric oxide production (NO) by macrophages is inhibited by LPG associated kinetoplastid membrane protein 11 which downregulates iNOS activity *(Bogdan et al, 1996: Jardim et al, 1995)*. A recent study has however demonstrated the evidence that internalization of *Leishmania* amastigotes displaying phosphatidyl serine (PS) on their surface induced the macrophages to produce IL-10 and TGFβ which blocked the induction of iNOS, thereby failing to generate NO *(Wanderley et al., 2006).* Similarly *Leishmania* infected macrophages do not produce superoxide. The NADPH oxidase enzyme is responsible for the production of superoxide, in case of infected macrophages the amastigote inhibits the protein kinase C (PKC) which further fails to phosphorylate p47 (a component of NADPH oxidase) thus failing to generate superoxide *(Lodge and Descoteaux, 2006)*. Responsiveness of *Leishmania* infected macrophage's to IFNγ is reduced either by decreasing the expression of IFNγ receptors on the surface or reduced phosphorylation of target proteins *(Nandan and Reiner, 1995; Blanchette et al., 1999)*. The Th1 response in case of *Leishmania* infection is compromised as IL-12 is not produced by the infected macrophages. The inhibitory effect of *Leishmania* in this case has been linked to JAK STAT signaling, while other studies have pointed out a role of ERK in the IL 12 production. Nuclear translocation of STAT1 is also inhibited in infected macrophages. Further amastigotes also inhibited nuclear translocation of NFκB which further inhibits IL 12 production by macrophages *(Cameron et al., 2004)*. Host immunity is severely compromised during infection as CD8+CD40+ T cells acting as contra Treg cells for a brief period of time gets destroyed by IL-10 mediated apoptosis *(Martin et al., 2010)*.

1.6 Extracellular vesicles of *Leishmania*

Recent research has made a striking revelation in which extracellular vesicles (exosomes) of *L. donovani* parasite contributes to the spread of infection **(Figure 1.2)**. These purified extracellular vesicles evoked a T helper 1 (T_H1) response in CD4+ T cells. These extracellular vesicles (EVs) are enriched in a virulence factor and surface metalloproteinase GP63 (known as leishmanolysin) *(Silverman et al., 2008; Hassani and Olivier, 2013; Hassani et al., 2014).* Injecting these EVs in mice enhanced the parasitic load. *Leishmania donovani* is known to invade hepatic cells and alter the lipid metabolism resulting in reduced choslesterol levels in circulating blood, this interference is mainly mediated by microRNA-122 (miR-122) based gene repression *(Ghosh et al., 2013).* The regulation is mainly brought about by GP63-Dicer 1- miR-122 axis as the GP63 degrades the Dicer1 and thus programmes miR-122 leading to alteration in lipid metabolism **(Figure 1.2)** *(Szempruch et al., 2016).*

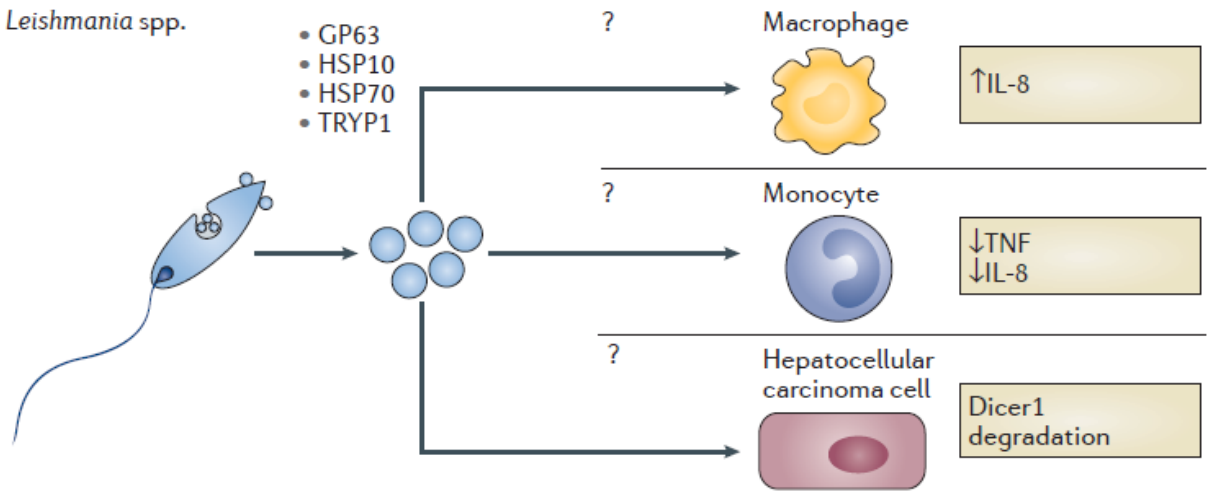


Figure 1.2. A novel mechanism adopted by *L. donovani* parasites for spreading the infection by budding of extracellular vesicles. (Image adopted from Szempruch et al**.,** 2016). The exosomes secreted by the parasite tend to alter the metabolic pathway of the host thus aiding in the appearance of the diseased condition.

1.7 Control measures and Diagnosis

Various control measures have been adopted in Indian subcontinent to minimize the spread of the disease for eg. spraying of DDT (dichloro-diphenyl-trichloroethane) in the 1950s

almost eliminated VL from Indian subcontinent as the sandflies were susceptible to DDT, since the discontinuation of DDT it has again reemerged. Also flies have started gaining resistance against continued use of DDT and other sprays. Emergence of HIV and VL coinfections has aggravated the whole issue. HAART therapy and the use of Liposomal amphotericin B have been recommended for such cases in the WHO Informal Consultative Meeting in Addis Ababa, 20–22 March 2007. *Phlebotomous argentipes* sandflies are responsible for spreading of VL in Indian sub continent. Several initiatives has been taken by Health Department which include integrated vector management, early diagnosis and complete treatment of cases, clinical or operational research as it is needed to reduce or completely eliminate the disease. Oral drug miltefosine along with a rapid diagnostic test rk39 immunochromatographic test has been recommended. Various diagnostic tests including rK39-based immunochromatographic test (ICT), direct agglutination test (DAT) and enzyme-linked immunosorbent assay (ELISA) are being employed for the detection of parasites. Another rapid and accurate version of DAT known as fast agglutination screening test (FAST) is now increasingly being used for detection purposes *(Schoone et al., 2001; Silva et al., 2005)*. In rK39-based test a 39 amino acid conserved repeat in protein kinesin is employed for ELISA based detection, the sensitivity of this test is 93-100%. The rK39-based test can be carried out on a test strip.

1.8 Treatment of Visceral leishmaniasis

1.8.1 Pentavalent antimonials

Pentavalent antimonials like sodium stibogluconate and meglumine have been used for the treatment of visceral leishmaniasis for over 70 years in Indian subcontinent. Their use however has been discontinued owing to emerging resistance in the *Leishmania* strains and huge toxicity issues like cardiac arrhythmia and acute pancreatitis *(Collin et al., 2004; Greenblatt et al., 1988)*. Pentavalent antimonials have been replaced by amphotericin B which forms the first line of defence, however it has its own toxicity related issues like fever, chills, rigor etc, also it is nephrotoxic. Use of pentavalent antimonials in India was initiated by Professor Brahmachari who synthesized urea stibamine against *kala azar* in 1920 *(Brahmachari et al., 1922, 1928)*. Synthesis of antimony gluconate (Solustibosan) was done in 1937 and sodium stibogluconate (Pentostam) in 1945 was reported by Brahmachari,

Schmidt, Kikuth, and others *(Kikuth and Schmidt., 1937, Goodwin., 1995).* Sodium gluconate (sodium stibogluconate or Pentostam) and meglumine antimoniate (Glucantime) are currently being used as antimony based compounds. It is injected intravenously or intramuscularly. Some common side effects of antimonials are anorexia, vomiting, abdominal pain, nausea, headache and lethargy. Despite their use as potent antileishmanials for more than 80 years still there is lack of clarity on the mode of action of these compounds. Earlier reports suggested an inhibition of fatty acid *β*-oxidation and glycolysis *(Berman et al., 1985).* Among the various models proposed for the mechanism of action prodrug model is widely accepted which postulates that pentavalent Sb(V) reduces to the active trivalent Sb(III) form in the amastigote stage *(Callahan et al., 1994),* although reduction in both stages of parasite has been reported. Thiols such as glutathione (GSH) and cysteine (Cys) aid in this reduction. Trypanothione (T(SH)2), a parasite specific thiol and Cys-Gly in the acidic compartment of mammalian cells has been predicted to be involved in the reduction process, but the rate of reduction has been reported to be slow. Entry of Sb (III) in the *Leishmania* parasite takes place through aquaglyceroporin (AQP1) *(Gourbal et al., 2004).* Few other studies have reported the enzymatic reduction of pentavalent Sb (V) by thiol-dependent reductase (TDR1) *(Denton et al., 2004).* Sb (III) mainly targets the trypanothione reductase (TR) and zinc finger protein. ($T(SH)_2$) is maintained in the reduced state by trypanothione system. Sb (III) inhibits the trypanothione reductase (TR) and thus interferes with the redox balance of the parasite resulting in the efflux of intracellular $T(SH)_2$ and GSH in the parasite *(Cunningham and Fairlamb., 1995).* Perhaps Sb (III) conjugated with $T(SH)_2$ is sequestered in vacuoles or excreted out through ABC (ATP binding cassette) transporters *(Mukhopadhyay et al., 1996, Légaré et al., 2001).* Basically the redox metabolism of the parasite is compromised on Sb (III) treatment. Type I topoisomerase is yet another target of Sb (V) *(Chakraborty and Majumder., 1988).* Several other studies suggest that SAG (Sodium antimony gluconate) induces the formation of ROS via PI3K-PKC-Ras/Raf-ERK1/2 pathway and NO by PI3K-Akt- p38MAPK- TNF*α*- iNOS2 axis in mouse macrophages and leads to parasitic death *(Mookerjee et al., 2006).* Upregulation of IFN-*γ* receptors in host derived monocytes has also been implicated in SAG mediated responses *(Dasgupta et al., 2003).*

Leishmania parasite has started gaining resistance to antimonials for the past 15-20 years in the Indian subcontinent as several resistance cases has been reported in the state of Bihar.

Therapy involving gradual increase in drug dosage is the chief cause of developing resistance in the parasite. According to one notion increased thiols like cysteine, GSH, and trypanothione in the parasite leads to antimony resistance as thiols can subvert the harms caused by antimony induced ROS and NOS *(Mukhopadhyay et al., 1996).* Resistant *Leishmania* parasites were rendered sensitive to antimony when treated with DFMO (DL-a-difluoromethyl ornithine) an ODC (Ornithine decarboxylase) inhibitor *(Grondin et al., 1997).* Other reports suggest an efflux mechanism in *Leishmania* parasite by MRP (Multidrug-resistance related protein) transporter *(Haldar et al., 2011).* Stibogluconate was also found to be an inhibitor of protein tyrosine phosphatase which causes an increase in cytokine response *(Pathak et al., 2001).* Another protein PRP1 has been implicated in the antimony based resistance *(Coelho et al., 2003).*

1.8.2 Amphotericin B

Owing to huge resistance and toxicity issues related to antimonials, they were subsequently replaced by Amphotericin B (AmB) drugs as a first line of defense for the treatment of VL. Liposomal formulations of amphotericin B have been used for the treatment of VL with efficacy *(Torrado et al., 2008).* It was initially isolated as a polyene antibiotic from *Streptomyces nodosus* in 1955 *(Dutcher et al., 1959).* Polyene hydrocarbon chain forms the hydrophilic domain while poly hydroxyl chain forms the hydrophobic domain which is mainly responsible for antifungal activity of amphotericin B *(Hamill, 2013).* It has been successfully used in the treatment of systemic fungal infections caused by *Candida albicans* and *Aspergillus fumigates* especially in the patients having compromised immunity such as AIDS. Lipid formulations of amphotericin B are administered by intravenous infusion. Amphotericin B usage involves complex treatment regimen (15 slow infusions on alternate days) and huge cost. The liposomal formulation of amphotericin B has been mainly recommended for VL. However amphotericin B has found limited use because of its nephrotoxicity which results in kidney failure *(Cohen., 1998; Lemke et al., 2005).* Various improved formulations of AmB like liposomes, emulsions and nanoparticles have come up to cope up with the adverse side effects *(Berman et al., 1992).* Various studies point out that AmB leads to the formation of ion channels rendering the parasitic membrane permeable which ultimately causes death by osmotic shock *(Houslay and Stanley, 2009).* Amphotericin

B is nephrotoxic as it affects the tubular cells of the kidney by increasing salt and Ca^{2+} concentration and its toxicity is dosage dependent thus low doses are generally recommended, however liposomal formulations of Amphotericin B in which deoxycholate has been replaced by lipids have been made which are less toxic *(Torrado et al., 2008).* These formulations are available in the trade name of L-AmB: Ambiosome, AmB colloidal dispersion (ABCD: Amphocil) and AmB lipid complex (ABL: Abelcit). Usage of liposomal AmB has shown 95% efficacy against VL. In a study it was found that the infection caused by *Leishmania* was inhibited when primary macrophages were treated with AmB, it was speculated that the AmB affected the membrane cholesterol content of macrophages thereby restricting parasitic entry. It is widely accepted that AmB binds to ergosterol in the membrane of *Leishmania* with high affinity *(Lemke et al., 2005).* The *in vivo* effect of AmB is however believed to be a combination of both host and parasitic effects as the AmB bind comparably with cholesterol and ergosterol *(Chattopadhyay and Jafurulla, 2011).* Amphotericin bound to cholesterol hinders the binding of parasite to the host cell membrane *(Paila et al., 2010).* Studies on AmB resistant strains of *L. donovani* have revealed that they lack C-24 alkylated ergosterol which is due to lower expression of SAMT (S-adenosyl methionine transferase). The recommended dose of liposomal amphotericin B for VL patients according to WHO guidelines for Indian subcontinent is 3-5 mg/kg per daily dose by infusion given over 3-5 days period up to a total dose of 15 mg/kg *(WHO guidelines 2015).*

1.8.3 Miltefosine

Miltefosine (hexadecylphosphocholine), now used for the treatment of VL was originally intended for its application in cancer. It was used as an anticancer drug in the 1980s and is effective against both visceral leishmaniasis and cutaneous leishmaniasis, however it is known to be teratogenic (embryotoxic). Miltefosine is the first registered oral drug to be used for the treatment of VL in India in 2002 (Impavido®; Zentaris GmbH) *(Croft and Engel, 2006).* It was synthesized as a structural analog of alkyllysophospholipid. The target regimen of this oral drug according to FDA approval is 2.5 mg/kg/day for 28 days, with a maximum dose of 150 mg/day *(Sindermann and Engel, 2006).* Miltefosine is readily available in the Indian subcontinent and is a good alternative for antimony resistant VL and CL. The molecular mechanism involved for the antileishmanial activity of miltefosine is debatable

and conflicting reports exists in literature. Various mechanisms for its action has been proposed like choline transport inhibition *(Zufferey and Mamoun, 2002)*, hindrance in the synthesis of ether-lipids and phospholipids with inversion of the phosphatidylcholine/ phosphatidylethanolamine ratio, mitochondrial dysfunction *(Getachew and Gedamu, 2012)* and cytochrome oxidase inhibition *(Luque-Ortega and Rivas, 2007)*. However the well accepted notion is that miltefosine induced cell death is due to mitochondrial dysfunction which leads to ROS generation. In 2004 Chinmoy Dey group reported that miltefosine induced cell death in the *Leishmania* parasite showed features of metazoan apoptosis which was supported by oligonucleosomal-DNA fragmentation and in situ TUNEL staining *(Verma and Dey, 2004; Paris et al., 2004)*. Further, gradual increase in concentration of Miltefosine and exposure for longtime leads to the development of resistance in the *Leishmania* parasite *(Moreira et al., 2011)*. Miltefosine can induce drug resistance in the parasite as it has long half life (150 hours), thus fears of emergence of resistant strains of the parasite cannot be precluded *(Perez-Victoria et al., 2006)*. Resistance to miltefosine arises mainly because of a mutation in one of its transporters LdMT (plasma membrane amino phopholipid translocase). To avoid the generation of drug resistance in parasites, combination therapy has been thought off as an alternative and drug combinations of miltefosine and liposomal amphotericin B are being tested in India *(Chappuis et al., 2007)*.

1.8.4 Paromomycin

Paromomycin (formerly known as aminosidine) is an antibiotic of aminoglycoside category which was introduced for the treatment of VL in the year 2006 and has shown promising antileishmanial effects *(den Boer and Davidson, 2006)*. It is produced from a bacterial pathogen *Streptomyces rimosus* var. *paromomycinus* (*Liu and Weller., 1996)*. It is a broad spectrum antibiotic effective against bacteria and protozoa *(Tracy et al., 2001)***.** Binding of paromomycin to 30S subunit of ribosome hinders with the protein synthesis machinery in the protozoan parasite *(Sundar and Chakravarty, 2008)*. Commercially it is available in the form of paromomycin sulfate with a brand name of Humatin. It is administered orally in the form of a capsule as well as intramuscularly in the form of an injection as it absorbs well through intramuscular route. In terms of efficacy it is almost equivalent to Amphotericin B and toxicity issues related with paromomycin usage are minimum. Continuous use of any of the

drugs poses threat for development of resistance in the parasite thus combination therapy involving two drugs is being used in the current times. A combination therapy involving paromomycin and miltefosine is currently in use. A combination therapy including paromomycin and sodium stibogluconate has been devised out in East Africa. It also acts against a plethora of pathogens including bacteria and has low cost (US$5–10 per treatment).

1.8.5 Pentamidine

Pentamidine belongs to diamidine class of drugs exhibiting antileishmanial properties and has been used as a second line of defense against visceral leishmaniasis. Pentamindine isethionate is given intramuscularly or preferably by intravenous infusion. Common adverse effects include diabetes mellitus, severe hypoglycaemia and shock myocarditis. It exerts its action by binding to DNA and also interferes with polyamine metabolism and disrupts mitochondrial membrane potential *(Bassalin et al., 2002).* Uptake of pentamidine in *Leishmania* parasite is by an unknown transporter *(Vercesi and Docampo, 1992).* Pentamidine has also been reported to be a comptetitive inhibitor of arginine transport and it inhibited putrescine and spermidine transport in a non competitive manner *(D'Silva and Daunes, 2000).*

1.8.6 Sitamaquine

Sitamaquine an 8-aminoquinoline analogue is another promising drug developed by Walter Reed Army Research Institute in collaboration with Glaxo Smith Kline (GSK) which is being tested in phase III clinical trials in India and Kenya. It can also be orally administered for the treatment of visceral leishmaniasis. Some studies have pointed out a transporter independent entry of sitamaquine in the parasitic cells *(López-Martín et al., 2008).* In a study it was proposed that sitamaquine enters the parasitic cells by diffusion *(Coimbra et al., 2010).* Sitamaquine is known to interact with the anioninc polar head groups of membrane phospholipids. Use of sitamaquine bears an advantage that it has a shorter half life (26 hr) as compared to miltefosine (150-200 hrs) thus the risk of drug resistance is eliminated.

1.9 Vaccines for Leishmaniasis

1.9.1 First generation vaccines

The observation that host becomes resistant to subsequent infections after recovery from primary infection gives the hope that vaccines can be made for the treatment of VL. Vaccines consisting of whole killed parasites or extracts have been made as first generation vaccines **(Figure 1.3)**. Many vaccines including *L. amazonensis* based vaccine in Brazil (Mayrink vaccine) and *L. maxicana* based vaccines in Venezuela (Convit vaccine) have been tested. The Convit vaccine is cost effective and safe to administer. The first generation vaccines in the form of live virulent parasites isolated from cutaneous lesions have been used in the Middle East and Russia in ancient times in a process called Leishmanization *(Nadim et al., 1983; Khamesipour et al., 2005).*

1.9.2 Second generation vaccines

Recombinant protein based second generation vaccines have also come up. Second generation vaccines have been the main focus in vaccine development in VL because they can address the genetic polymorphism of mammalian immune system in a better way. One such second generation vaccine Leish-111f + MPL-SE is now in clinical trial. Its evaluation is being done in phase-1 trial in India and in phase-1-2 trial in Peru. In several reports it was shown that protection from VL was conferred when animal models such as mice and dogs were vaccinated with fucose-mannose ligand *(Gamboa-Leon et al., 2006; Martiny et al., 1999). Leishmania donovani* promastigotes membrane antigens entrapped in cationic liposomes have also been shown to confer immunity against VL in mice and hamsters *(Afrin and Ali, 1997; Ali and Afrin, 1997; Afrin et al., 2000, 2002).* Soluble leishmanial antigen (SLA) entrapped in cationic liposomes also showed prophylactic and therapeutic efficacy against VL *(Bhowmick et al., 2007).* Another fraction belonging to 97.4-68 kDa from *L. donovani* promastigotes stimulated Th1 type cellular response in cured VL patients and hamsters *(Garg et al., 2006).* Many subunit vaccines have also been tested for eg. LPG provided protection against CL *(Handman and Mitchell, 1985; McConville et al., 1987; Karanja et al., 2011).* Surface expressed glycoprotein leishmaniolysin (gp63) is conserved across all VL species such as *L. donovani, L.infantum* and *L. chagasi*, and thus has been used as a vaccine candidate. Recombinant gp63 failed to initiate substantial immune response but

liposomal delivery of recombinant gp63 generated good immune response against both VL and CL *(Mazumder et al., 2011)*. Carboxy terminal domain (CTD) of Histone H2B protein is also conserved across all VL species and thus has proved to be antigenic and can be used for cross species protection *(Meddeb-Garnaoui et al., 2010)*. Another subunit vaccine comprising of ubiquitin conjugated ORFF protein of *L. donovani* has shown protective efficacy against VL *(Sharma and Madhubala, 2009)*.

Multicomponent or polyprotein vaccines have shown better efficacy for eg: LEISH-F1 (formerly Leish111f) formulated by The Infectious Disease Research Institute (IDRI, Seattle, WA) comprises of *Leishmania* elongation initiation factor (LeIF), thiol-specific antioxidant (TSA), and *L. major* stress-inducible protein-1(LmSTI1). This multicomponent vaccine conferred immunity against *L. major* and *L. infantum (Coler et al., 2007)*. LEISH-F1 is the first vaccine to enter phase I and II clinical trials. After the successful phase I trials of LEISH-F1, IDRI developed LEISH-F2 and entered into phase I and II clinical trials. Another polyprotein vaccine termed KSAC comprising of four proteins: SMT, A2, CPB and kinetoplastid membrane protein-11 (KMP-11) was found to be immunogenic *(Goto et al., 2011)*.

1.9.3 Third generation vaccines

Third generation vaccines are based on DNA vaccines which elicit a stronger Th1 response. *Leishmania* analogue of the receptor kinase C (LACK), gp63 and ORFF have been used as DNA vaccine candidates. Further when a combination of plasmids harboring gp63 and Hsp 70 genes were delivered to mice it elicited strong Th1 response *(Kaur et al., 2011)*. In another set of interesting studies cDNA libraries from amastigote RNA were prepared and used for immunization of BALB/c mice and candidate genes for DNA vaccinations were identified *(Melby et al., 2000)*. LEISHD- NAVAX, which is in preclinical trials in Europe, has been developed by Mologen AG (Berlin, Germany) for cutaneous and visceral forms of leishmaniasis. Another DNA vaccine called NH36-DNA vaccine was tested against *L. chagasi* in BALB/C mice and significant reduction in parasite load was found *(Gamboa-Leon et al., 2006)*. Several other DNA vaccines like KMP-11, N terminal domain of PPG have been tested successfully.

1.9.4 Vaccines based on sandfly saliva

Another interesting observation made in this context is that individuals exposed to infected sandfly gained some immunity against VL, thus many salivary and insect gut proteins have been used as vaccines. PpSP15, maxadilan and LJM17 proteins from insect saliva have been reported as potent immunogens which showed upregulation of IFN-γ and IL-12. LMJ19 is such a protein from *Lutzomyia* saliva which conferred immunity against VL as well as in CL in hamsters *(Gomes et al., 2008)*.

1.9.5 Live attenuated vaccines

In an attempt to make live attenuated vaccine a null mutant of centrin (Ld Cen-/-) has been prepared and is mentioned to protect BALB/c mice and Syrian hamsters *(Selvapandiyan et al., 2009)*. Another biopterin transporter null mutant (BT1-/-) generated Th1 response with increased levels of IFN-γ and IL12 *(Papadopoulou et al., 2002)*. Strong IgG response was generated in a study when one allele of SIR2 in *L. infantum* was mutated. In a completely different approach a non pathogenic *L. tarentolae* was used as a live vaccine and displayed cross species protection *(Mizbani et al., 2009, 2011)*. Another knockout mutant of Ldp27 (−/−) reported by Dey et al stimulated the CD4+ and CD8+ population of T cells *(Dey et al., 2013)*. The risk associated with the use of live nonpathogenic and genetically modified forms of *Leishmania* as vaccines is that it can revert back to their virulent form. Most of the vaccines are in clinical trials and none of them have been licensed yet for use.

1.9.6 Synthetic polyvalent peptide vaccines

After the successful testing of many forms of vaccines, an idea has been strengthened that some amino acid sequences in proteins mainly elicit the immune response. Thus synthetic polyvalent peptide vaccines have been developed. Epitope interactions and epitope mapping have been utilized for the development of such vaccines. Many epitopes from GP 63 have been identified which induce Th1 response and has established GP63 as a potential vaccine candidate. In a similar way thirty peptides from KMP 11 protein were assessed for their ability to induce IFN-γ production by CD8+T cells *(Basu et al., 2007)*. Enzymes involved in redox metabolism of the parasite have always been targeted as drug candidates, they have also been utilized as vaccine candidates such as pVAXγGCS (gamma-glutamyl cysteine

synthetase) and UBQ-ORFF which also elicited IL-12 and IFN-γ production indicating Th1 response *(Carter et al., 2007)*. Several membrane embedded proteins as well as soluble proteins from *L. donovani* promastigotes have been investigated as putative recombinant protein vaccines for their role in eliciting antigenic response. LCR1 and HASPB1 are membrane proteins which elicited good response against visceral leishmaniasis *(Wilson et al., 1995)*. In a recent report a hypothetical protein (LiHyp1) elicited IFN-γ production by CD4+ T cells via IL-12 *(Martins et al., 2013)*.

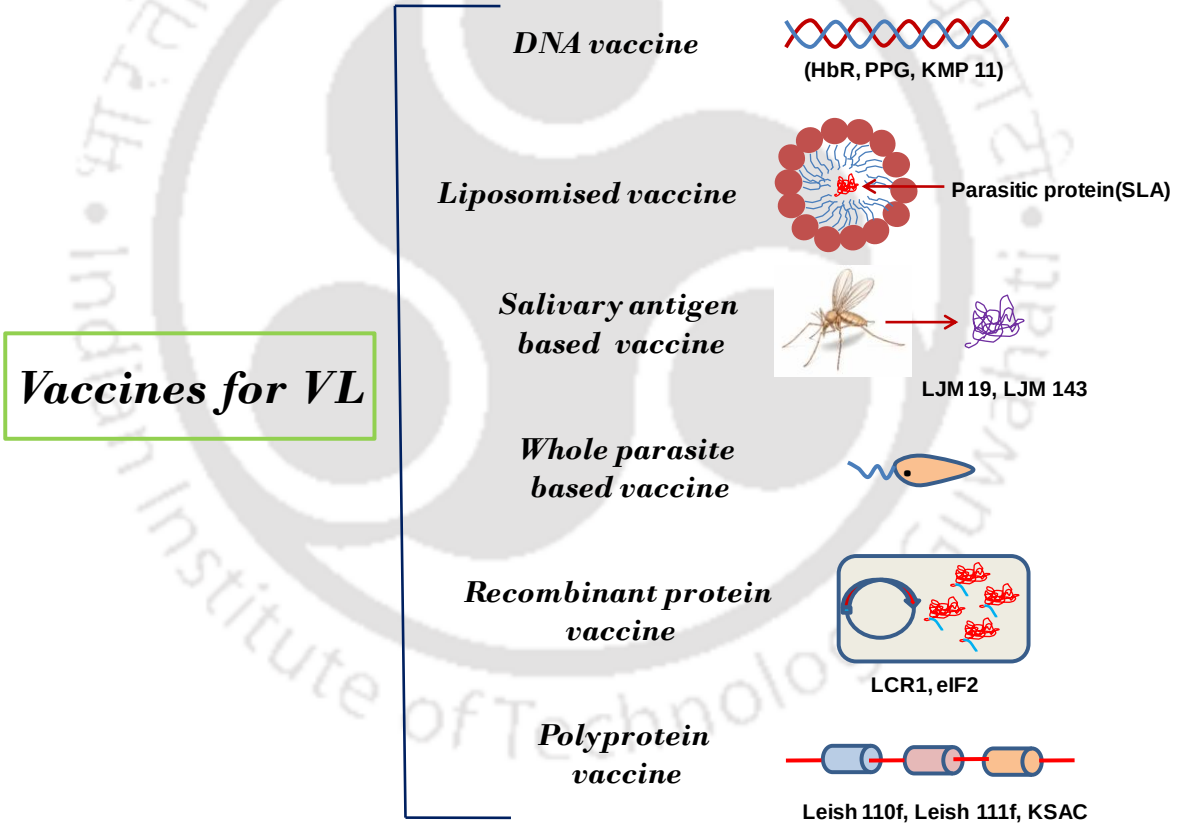


Figure 1.3. A schematic representing the development of vaccines for visceral leishmaniasis. Various vaccines like DNA vaccine, liposomised vaccine, salivary antigen based vaccine, whole parasite based vaccine, recombinant protein vaccine and polyprotein vaccines have been tested for their efficacy against visceral leishmaniasis.

1.10 Isolation of *Leishmania*

In the case of visceral leishmaniais, the *Leishmania donovani* are usually isolated from bone marrow and splenic aspirates. Cultivation is usually done in a blood agar medium (Novy-MacNeal-Nicolle (NNN)). *Leishmania donovani* can be isolated from dogs using lymph nodes. Parasites can also be isolated directly from the gut of sandflies. Sandflies are rinsed with saline and guts are dissected out and the contents visualized under microscope for the presence of promastigotes. The gut contents can be directly cultured or inoculated into hamster. Parasites can be reisolated from the hamster later. Usually the snout, feet or peritoneal cavities of hamsters are inoculated with the positive gut contents. Various laboratory animals like golden hamsters, inbred and outbred BLAB/C mice are utilized for the inoculation purposes. Intradermal inoculation of dermatotropic *Leishmania* in nose and rear feet is usually preferred for golden hamsters while peritoneal route is preferred for visceral leishmaniasis. Animal is killed within 7-14 days and smears prepared from spleen and liver. Various commonly used blood agar based biphasic media such as NNN medium, USAMRU medium, Evans modified Tobie medium and Schneider drosophila medium are used for *Leishmania* culture. *Leishmania* are usually preserved at -80°C or in liquid nitrogen at -196°C *(WHO report, 2012)*.

1.11 Organization of control

Leishmaniasis is endemic in about 98 countries and a large chunk of population in the third world countries has poor access to health care facilities. As this disease is prevalent in the poor, thus economic status has played a major role. Financial constraints and the distance of health care units also play a major role. Various control measures are being taken to prevent the spread of leishmaniasis at various levels. Living conditions of the populations residing in endemic areas should be improved. Also the cost involved in the treatment of the disease and drugs involved should be reduced, so that a wider population can afford the treatment. Various tasks have been distributed to carry out this effectively which include identification of clinically suspected areas, sites of high transmission and control of animal reservoir host. Primary health care units have been assigned this task which also includes diagnosis, treatment as well as follow up. District health office is concerned with developing and implementing a plan, implementing vector control and investigating outbreaks. Availability

of skilled personnel, better facilities and finances are must in endemic countries for functioning of proper control measures. The committee should ensure that follow up examinations are being attended by patients. Proper training of health care workers and introduction of rapid diagnostic tests like rK39 is mandatory. Laboratory technicians should be well versed with various diagnostic kits. Training of community health workers should help them in the identification of VL cases. A control manual describing various methods and control techniques should be available. National control programmes of government should be made with clear objectives, methods and schedules. Cost effectiveness and feasibility of the project should be determined. Monitoring and evaluation is a vital part of any National control programme which includes prevalence, incidence and geographical boundaries. Progress should be measured regularly. World health organization (WHO) has come up with various control strategies and has established regional centers to monitor the situation closely. Surveillance should include proper collection of data and dissemination of data. Health care centers should help in data collection such as number of visceral leishmaniasis cases in a particular area and should take appropriate preliminary actions such as indoor spraying etc.

In many cases patients do not comply with the treatment regimens owing to toxic side effects, thus pharmacovigilence needs to be strengthened in *Leishmania* endemic countries. A routine monitoring of the emerging parasitic resistance to newer medicines should be done. Severe events related to the use of drugs for leishmaniasis should be brought into light. Collaboration of National drug regulatory agencies and National control programme is essential in prevention and curing of disease. On the basis of rates of morbidity and mortality the control programmes should evaluate and revise their objectives with proper deadlines. A national surveillance system usually collects the data. International coordination, non government organizations and research institutions should involve more so as to accelerate the curbing of the disease. Emergency preparedness at international level is essential in times of difficulty and epidemic outbreak. Laboratory research should focus more on the development of vaccines. There is a need for the improvement of detection techniques based on the molecular biology of the parasite. As the treatment options for PKDL are limited, so immunotherapy and combination treatment can be used for it. Similar combination treatment strategies should be developed for *Leishmania*-HIV co infection. For the validation of new

drugs *in vivo* testing of lead molecules and their conversion to candidate drugs should be done in order to assess "druggability" and "drug likeness" of these lead compounds.

1.12 Leishmaniasis elimination programme

A kala-azar elimination program has been chalked out by the World helath organization (WHO) for three south Asian countries endemic to leishmaniasis: India, Nepal and Bangladesh. The elimination program will comprise of 4 phases (1) Preparatory phase (2) Attack phase (3) Consolidation phase, and (4) Maintenence phase. The preparatory phase ensues after the agreement of three countries on the overall plan. During this phase major guidelines for the implementation programme are laid out. Policy making is also an important part of this phase. The actual implementation of the programme begins in the attack phase during which affected areas are identified and integrated vector management (IVM) and indoor residual spraying (IRS) is applied in the affected areas for 5 consecutive years. Conclusion of IRS marks the beginning of consolidation phase which takes around 3 years to complete. Recurrence in cases is noted down during this phase and early treatment is provided. Finally in the maintenance phase the surveillance is continued. During this phase the cases should drop to 1 per 10,000. Since the inception of this programme the number of cases has dropped to significant levels *(WHO Report, 2012).*

1.13 Biosynthesis of pyrimidines

Two branches of pyrimidine pathway: *de novo* pyrimidine biosynthetic pathway and salvage pathway led to the synthesis of pyrimidines which are important constituents of DNA and RNA as well as of UDP sugars and CDP-lipids (CDP-choline). Nucleotide derivatives are important constituents of vitamins as well. Uridine nucleotides also regulate physiological processes by acting at extracellular receptors *(Connolly and Duley, 1999)*. The *de novo* pathway synthesizes pyrimidines from the basic units like L-glutamine, bicarbonate and aspartate. Glutamine contributes the amide nitrogen of a pyrimidine ring while the carbon skeleton is contributed by aspartate and bicarbonate. Ribose (synthesized via pentose phosphate pathway) is later added to the pyrimidines to constitute a pyrimidine nucleotide, while salvage pathway synthesizes the pyrimidines from preformed precursors (preformed nucleobases and nucleosides) "salvaged" out from the cell or external medium by

transporters. Resting cells mostly rely on salvage pathway while *de novo* pathway slows down *(Fairbanks et al., 1995)*. While *de novo* pathway takes over salvage pathway in proliferating cells, thus *de novo* pathway garners wider interest as it is concerned with growing cells and is subject to therapeutic intervention. In *Leishmania donovani* both pathways are functional leading to pyrimidine synthesis while in a related parasite *Plasmodium falciparum* only *de novo* pathway is functional. The *de novo* pathway has been of prime interest as the enzymes involved in the *de novo* pathway are regulated to orchestrate the cell division *(Jacobson and Stark, 1973)*. The pyrimidine pathway is linked to purine pathway by phosphoribosyl pyrophosphatase (PRPP). Pyrimidines are not only the vital constituents of nucleic acids but also act as sugar and phospholipid carriers.

De novo pathway functions by synthesizing uridylic acid by a series of six enzymatic steps. The pyrimidine pathway is fine tuned by enzyme regulations. The initial enzyme carbamoyl phosphatase is regulated by feedback inhibition by the UTP and CTP (end product) in an allosteric manner. The first cyclic product: dihydroorotate of the pathway appears when the third step is catalyzed by dihydroorotase utilizing N-carbamoyl aspartate as a substrate. In animal cells the first three steps are catalyzed by a single multifunctional enzyme referred to as CAD (Carbamoyl phosphate synthetase, Aspartate transcarbamoylase, Dihydroorotase). The three enzymes in a CAD complex are arranged as: NH_3^+-CPSase-DHOase-bridge-ATCase-COO^-. The CAD complex is also found in fungi, amoebozoa and red alga *Cyanidioschyzon merolae*. The first three enzymes are also known to form a complex in *Trypanosoma cruzi* as all the three enzymes co immunoprecipitated *(Nara et al., 2012)*. The three enzyme activities are assigned to a single 243 kDa polypeptide which further reconstitutes to form hexamers and higher order oligomers forming a 1.4 MDa complex, roughly half the size of a ribosome *(Lee et al., 1985)*. While in *Leishmania donovani* three independent enzymes catalyze the initial three steps, which bring us to the conclusion that there is major structural difference in the initial steps of the pathway between parasite and host.

Pyrimidines are also supplied to the cell through salvage pathway which involves enzymes like uridine phosphorylase, uridine kinase, cytidine deaminase and thymidine kinase. Salvage pathway involves a rate limiting step which converts uridine to UMP as this step is inhibited by CTP and UTP.

In the bacterial system since the same CPSase is utilized for pyrimidine as well as arginine biosynthesis, thus ATCase is the first commited step in pyrimidine biosynthesis in bacteria and thus subjected to regulation *(Lipscomb, 1994)*. While the mammalian cells have two CPSase: CPSI for urea biosynthesis and CPSII for pyrimidine biosynthesis *(Lusty, 1978)*. Thus in mammalian cells CPSII catalyzes the first step of the pathway and ATCase is unregulated. The fourth step of the pathway is catalyzed by dihydroorotate dehydrogenase, an inner mitochondrial membrane enzyme. The last two steps of pyrimidine biosynthesis are carried out by a bifunctional enzyme UMP synthase which comprises of 24 kDa orotatephosphoribosyltransferase (OPRTase) and a 28 kDa orotidine-5'-phosphate decarboxylase (OMPdecase) *(Suttle et al., 1988)*. The UMP formed in the pyrimidine pathway is further phosphorylated to generate UTP to which amide nitrogen of glutamine is added at the C4 position to generate CTP by CTP synthase (CTPSase) *(Zalkin, 1985)***.** Except dihydroorotate dehydrogenase all the enzymes of *de novo* pyrimidine pathway are localized in the cytoplasm. The CAD is shown to have proximity to the cytoskeleton which probably aids this multifunctional protein complex to slide to mitochondria where dihydroorotate dehydrogenase is localized thus aiding in efficient functioning of the pathway *(Christopherson and Jones, 1980)*.

Expression of CAD genes is enhanced when cell needs more supply of pyrimidines like in proliferating cells. CAD expression levels are regulated transcriptionally and post transcriptionally. When the cells enter G1/S phase Myc binds to E box region upstream of CAD genes and increases the expression of CAD genes *(Boyd and Farnham, 1997; Khan et al., 2003)*. CAD is also regulated at the protein level as the caspase binding causes the degradation of CAD at the initiation of apoptosis *(Huang and Graves, 2003)*.

1.14 Regulation of *de novo* pyrimidine pathway in mammalian cells

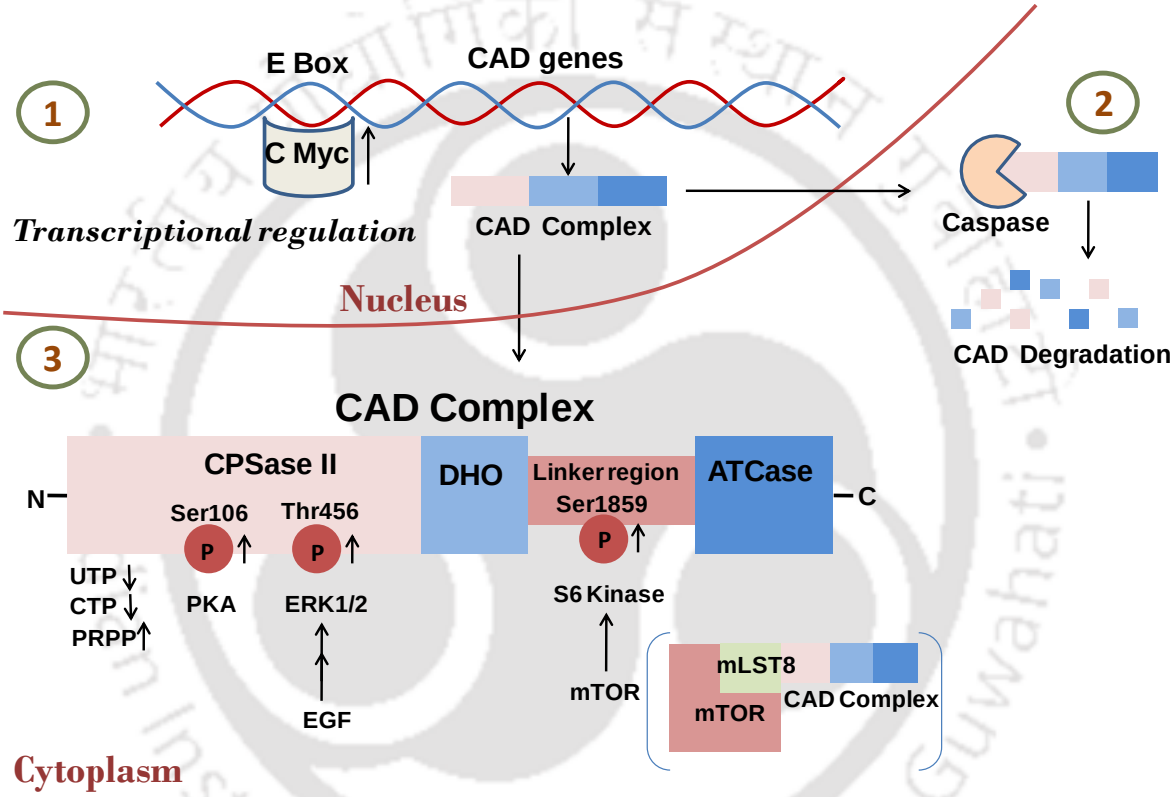
In the mammalian cells CPSase, the first enzyme of the pathway is subject to regulation by UTP which regulates CPSase by feedback inhibition **(Figure 1.4)**. It is also activated in an allosteric fashion by PRPP. Another way in which CAD complex is regulated is by phosphorylation of a Ser-106 residue by PKA (protein kinase A) which decreases the affinity of CAD to UTP and thus leads to enhanced pyrimidine production in the cell *(Carrey et al., 1985)*. Another phosphorylation event on CPSase turns the role of UTP to that of an

activator, thus increasing pyrimidine production. This event is brought about by ERK1/2 mediated phosphorylation of Thr-456 residue on CPSase in response to epidermal growth factor signaling *(Graves et al., 2000)*. A more clear picture on regulation of CAD complex emerged in the year 2013 breakthrough publication in which CAD complex was shown to be activated by Ser-Thr kinase mTOR based phosphorylation of Ser-1859 via S6 Kinase. Ser-1859 site is situated in the linker region that connects the aspartate transcarbamoylase and dihydroorotase domains in the CAD complex in mammalian cells. Ser-1859 phosphorylation mark on CAD helped in the progression through S phase of cell cycle by enhancement in pyrimidine pool synthesis *(Robitaille et al., 2013)*. A S1859A mutation rendered CAD insensitive to mTORC1 S6K1 mediated phosphorylation. Phosphorylation events on Thr-456 and Ser-1859 act independently of each other. Abundance of N-carbamoyl aspartate (dihydroorotase substrate) also increased in mTORC1 treated cell lines. mTORC1 also increased the pyrimidine incorporation in the DNA and RNA *(Ben-Sahra et al., 2013)*. In another study it was reported that CAD complex interacted with the mTOR via mLST8 a component of mTOR complex *(Nakashima et al, 2013)*. Mammalian CAD complex also immunoprecipitated with Rad9, a DNA checkpoint protein. Rad9 stimulates the activity of CPSase of CAD complex by 2 folds. The N terminal domain of Rad9 interacted with CPSase only when Rad 9 was not a part of 9-1-1 checkpoint complex *(Lindsey-Boltz et al., 2004)*. This explains that the pyrimidine metabolism is also regulated by DNA repair pathways. A detailed schematic representing the regulation of *de novo* pyrimdine biosynthesis pathway can be found in **(Figure 1.4)**. CTPase which controls the UTP and CTP pools is subjected to allosteric activation by GTP and inhibition by its end product CTP, any kind of phosphorylation events for this enzyme are unheard off *(Van Kuilenburg et al., 1998; Evans and Guy, 2004)*.

1.15 Regulation of *de novo* pyrimidine pathway in *E.coli*

In *E.coli* the open reading frames of all pyrimidine genes are arranged in the form of an operon referred to as *pyr* (pyrimidine) (*carA*, *carB*, *pyrB*, *pyrC*, *pyrD*, *pyrE*, and *pyrF*). However they are not regulated in an activator repressor type system, instead they follow a more complex regulation at the level of transcription as that observed for tryptophan operon i.e they sense the availability of pyrimidines through RNA polymerase and leader sequences

in their transcripts and accordingly regulate the synthesis. An mRNA binding regulatory protein PyrR regulates the synthesis of *pyr* operon transcripts *(Turnbough and Switzer, 2008)*. However in *Mycobacterium smegmatis* the mRNAs for *pyr* operon lacks the leader regions thus PyrR regulates at the level of translational repression by occluding the first ribosome binding site *(Fields and Switzer, 2007)*.



Regulation of de novo pyrimidine synthesis

Figure 1.4: Schematic representation of the regulation of *de novo* pyrimidine biosynthesis pathway **(1)** Transcriptional regulation of CAD genes by binding of C myc to E Box upstream of CAD genes. **(2)** Caspase mediated degradation of CAD complex on the onset of apoptosis. **(3)** Regulation at the protein level by phosporylation events at Ser 106, Thr 456, Ser 1859 by PKA (Protein Kinase A), ERK1/2 and mTOR respectively which enhances pyrimidine production. Feedback inhibition by UTP, CTP (end products) and stimulation by PRPP.

In *E. coli* dual regulation of the *de novo* pyrimidine pathway takes place. Firstly it is regulated by classical feedback inhibition of ATCase by the end products CTP and UTP, secondly it is regulated by another mechanism referred to as directed overflow metabolism in which the near terminal pathway enzyme uridine monophosphate kinase is regulated in a cooperative feedback manner. This leads to a buildup of uridine monophosphate which is degraded by a phosphatase known as Umph. This dual regulation of the pathway in *E. coli* maintains the homeostasis in the pathway *(Reaves et al., 2013)*.

1.16 *De novo* pyrimidine pathway mediates viral growth

A recent study has pointed out a connection between inhibition of pyrimidine pathway and antiviral response via innate immunity. The antiviral response was initiated when dihydroorotate dehydrogenase was inhibited by DD264 *(Lucas-Hourani et al., 2013)*. In a recent interesting finding it was found that Human cytomegalovirus (HCMV) viral protein glycosylation relies on host pyrimidine biosynthesis and a hindrance in the host pyrimidine biosynthesis caused significant decrease in viral loads. The HCMV virus depends on the continuous supply of UDP-sugar synthesized by *de novo* pyrimidine pathway to glycosylate its proteins. HCMV somehow modulated the pyrimidine biosynthesis by regulating the activity of CAD by either MAPK (phosphorylation of Thr-456 on CAD) pathway or by mTORC1–S6k (phosphorylation of Ser- 1859 on CAD) which enhanced the pyrimidine pools. The glycosylation of viral envelope glycoprotein B (gB) was attenuated when pyrimidine biosynthesis was inhibited *(DeVito et al., 2014)*. In a similar study dengue virus was inhibited when the host *de novo* pyrimidine pathway was inhibited by brequinar which inhibits DHOD *(Wang et al., 2011)*.

1.17 Multifaceted roles of *de novo* pyrimidine pathway

The role of *de novo* pyrimidine pathway and dihydroorotase has been reported in various studies. In one such study in the fungus *Cryptococcus neoformans* the dihydroorotase null mutants (URA4 mutants) displayed uracil/uridine auxotrophy as expected, but also were slow growing and had weakened infectivity. The survival rate of *C. neoformans* (URA4 mutants) in the macrophage was also decreased *(de Gontijo et al., 2014)*. Similar reduction in growth was also reported for *Salmonella typhimurium* pyrC mutant (*pyrC* and *ura4* code for

dihydroorotase in *S. typhimurium* and *C. neoformans* respectively) *(Yoshikawa et al., 2011)*. In yet another striking revelation the role of pyrimidine pathway in biofilm formation by *E. coli* was reported. The capability to form a biofilm in *E. coli* depends on several factors like extracellular polysaccharide production which is closely regulated by curli operon system and production of cellulose which was shown to be strongly dependent on the availability of pyrimidines. Curli production in *E. coli* was abolished when the UMP biosynthetic genes were inactivated *(Garavaglia et al., 2012)*. A recent publication in Nov. 2016 has reported that *de novo* biosynthesis of pyrimidines depends on pyrimidine salvage enzymes in *Trypanosoma brucei (Leija et al., 2016)*. Though all the six enzymes of *de novo* pyrimidine pathway have been reported to be solely functional for pyrimidine synthesis, but a recent report has suggested an additional unidentified role for the fourth enzyme dihydroorotate dehydrogenase in the *Toxoplasma gondii* parasite, as the genetic ablation of the fourth enzyme did not yield uracil auxotrophy. Dihydroorotate dehydrogenase is also emerging as a potential drug candidate for the treatment of malaria, and it points out to a wider role played by this enzyme other then pyrimidine synthesis *(Hortua Triana et al., 2016)*. There is a growing line of evidence which suggests that pathogens interfere with the purine and pyrimidine metabolic pathways of host. A whole cell metabolic analysis in *Staphylococcus aureus* infected human airway epithelium cell line showed drastic decrease in *de novo* pyrimidine biosynthesis which was also confirmed by labeling experiments which supported reduced uptake of precursors *(Gierok et al., 2016)*.

One the one hand viruses like HCMV increases the host pyrimidine pool for their benefit while on another side some bacteria like *S. aureus* tend to decrease the pyrimidine pools in the host cells thus impairing basic functions. The way in which pathogen can influence the *de novo* pyrimidine pathway can be dramatic. Pyrimidine starvation in cells could also lead to apoptosis as was revealed by a metabolic screen for myeloma cells treated with AICAr (5-aminoimidazole-4-carboxamide-1-β-riboside) *(Bardeleben et al., 2013)*.

1.18 Pyrimidine degradation

Pyrimidine degradation pathways have been well described in *E. coli*. Uracil and Thymine in the *E. coli* are degraded by two different pathways termed as reductive and oxidative pathways each consisiting of three steps. Uracil or thymine degradation via reductive

pathway leads to the formation of β-alanine along with NH_3 and CO_2 while the oxidative pathway leads to the formation of urea along with NH_3 and CO_2. Another degradation pathway has been discovered in the past decade that leads to the formation of 3-hydroxypropionic acid along with NH_3 and CO_2. This newly discovered pathway is regulated by b1012 operon in *E. coli.* K12 also known as *rut* operon (pyrimidine utilization) (*rutABCDEFG*). However this particular degradation pathway is absent in *Salmonella typhimurium.* Pyrimidine degradation pathways in a cell serves many purposes like extracting out the nitrogen from the nitrogen rich pyrimdines, synthesis of β-alanine and it also checks the extra supply of pyrimidines in starving conditions and helps in maintaining a balance in metabolism of pyrimidines *(Loh et al., 2006).* Similar pyrimidine degradation pathway has also been identified in *Drosophila melanogaster (Rawls JM Jr, 2006).* Oxidative pathway has not been reported in fungi but reductive pathway involving URC pathway has been reported in *L. kluyver (Andersson Rasmussen, 2014).*

1.19 Pyrimidine pathway in *Leishmania donovani*

Kinetoplastids possess the ability to synthesize pyrimidine ring *de novo* using glutamine and aspartate as precursors while they can also salvage preformed nucleosides and nucleobases **(Figure 1.5)** *(Ali et al., 2013).* But still it is interesting to find out which particular pathway is more essential to the parasites.

In the quest for novel drug targeting enzymes nucleotide pathways offered an interesting area to look for. It was subsequently revealed that pathogenic protozoans have lost the purine biosynthetic pathway but retained the salvage pathway. This triggered a series of inhibitor based approaches based on purine pathway but the presence of complex interconversion pathways makes this approach a difficult one *(Ali et al., 2013).* All parasitic protozoans can synthesize pyrimidine ring as well as are capable of salvaging them. *Plasmodium falciparum* is defunct in pyrimidine salvage and relies on synthesis of the same *(Ali et al., 2013).*

Leishmania donovani has a functional *de novo* and salvage pyrimidine biosynthetic pathway i.e the parasites are prototrophic to pyrimidines while they are auxotrophic to purines since they are deficient in purine biosynthesis and can only salvage purines *(Boitz et al., 2012). L eishmania donovani* can synthesize pyrimidines *de novo* while they can salvage

preformed pyrimidines from the extracellular environment *(Wilson et al., 2012).* A schematic of *de novo* pyrimidine pathway of *L. donovani* is being presented in **Figure 1.5.** The *de novo* pathway comprises of six enzymes working in a cascade to produce UMP which is further phosphorylated to produce UDP and UTP which finally gets converted to CTP. In a study carried out by Wilson et al they showed that knocking out *cps* gene conferred auxotrophy while knocking out *cps* along with *uprt* lead to death of parasites. The arrangement of pyrimidine biosynthetic genes is syntenic (on the same locus) i.e on the 16^{th} chromosome of the parasite while this synteny is lost in the case of *Trypanosoma brucei* at *dho* locus. However the salvage pathway genes do not exhibit any synteny in their arrangement *(Wilson et al., 2012).*

Knockout of last two enzymes Orotidine Monophosphate Decarboxylase (*PYR6*, OMPDCase) and orotate phosphoribosyltransferase (*PYR5*, OPRT) of pyrimidine *de novo* biosynthetic pathway in *Trypanosoma brucei* established the essentiality of *de novo* pathway in in vitro conditions only while in *in vivo* conditions parasites could retain their viability by salvaging extracellular pyrimidines *(Ali et al, 2013).*

Although the *de novo* pyrimidine biosynthetic pathway is well conserved across the animal kingdom, still the mammalian pathway differs from that of *L. donovani*'s pathway in many aspects. Firstly, the first three enzymes of the mammalian pyrimidine biosynthetic pathway are clubbed together in the form of trifunctional enzyme referred to as CAD while the parasite has three independent enzymes for the initial three steps of the pathway **(Figure 1.5)** represents the *de novo* pyrimidine pathway in *L. donovani*). Secondly the localization of *Ld* DHOD is cytoplasmic while the mammalian counterpart localizes in the inner mitochondrial membrane with dependence on ubiquinone. Since *Ld*DHOD is cytoplasmic it requires fumarate for its functioning rather then ubiquinone. Thirdly, the last two enzymes of the pathway OPRT and OMPDC are in the form of a bifunctional enzyme in both parasite as well as in the host counterpart but their order being reversed in the parasite as they are arranged in the sequence of OMPDC-OPRT, while this arrangement is reversed in the mammalian counterpart. This crucial difference in the arrangement of last two enzymes was exploited in a study in which the essentiality of *Ld*UMPS (bifunctional enzyme consisting of OPRT and OMPDC domains) was demonstrated in *L. donovani* and it was deduced out that it was essential for parasite survival *(Jarrod et al., 2011).* Fourthly, the mammalian OPRT-

OMPDC is localized cytoplasmically while the parasitic counterpart most probably localizes to the glycosome which the cell fractionation studies have pointed out. The regulation of *de novo* pyrimidine pathway in *L. donovani* is slightly different from the mammalian counterpart. The CPSase of *L. donovani* is inhibited by UDP but not activated by PRPP *(Carter et al., 2008)*. A comparison of pyrimidine pathway in *L. donovani* and mammalian cells is presented in **Figure 1.6.**

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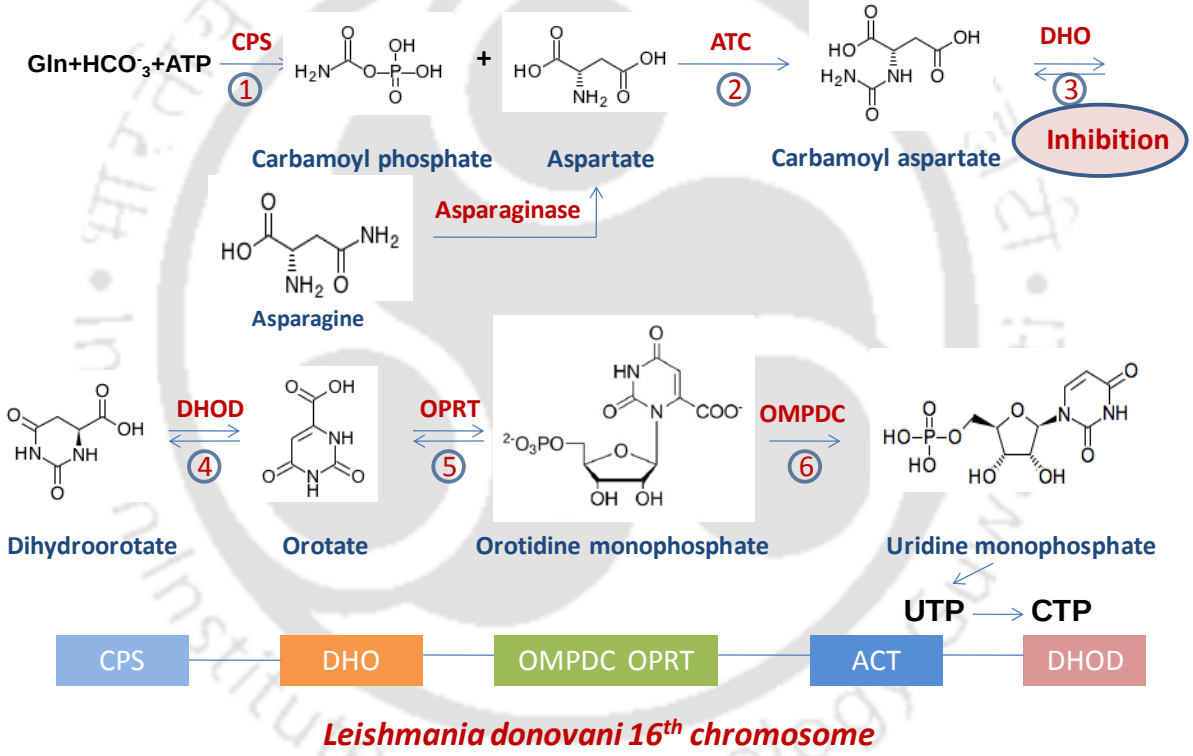

Figure 1.5: *De novo* pyrimidine biosynthesis pathway in *Leishmania donovani*. Also showing syntenic arrangement of *de novo* pyrimidine biosynthesis genes on 16th chromomome of *L. donovani*. CPS: Carbamoyl Phosphate Synthetase, ATC: Aspartate Transcarbamoylase, DHO: Dihydroorotase, DHOD: Dihydroorotate Dehydrogenase, OPRT: Orotate Phosphoribosyl transferase, OMPDC: Orotidine 5' Monophosphate Decarboxylase.

Leishmania donovani has a well defined salvaging capacity for pyrimidines by virtue of its various transporters like NT1 which transports pyrimidine nucleosides. Another transporter LmU1 has been described in *L. major* which identifies uracil. The glutamine analog avicin which inhibits CPSase leads to the death of the amastigote and promstigote forms of the parasite *(Poster et al., 1981)*. In the same manner N-(phosphonoacetyl)-L-aspartic acid (PALA) a specific inhibitor of ATCase inhibited the growth of both life forms of the *L. donovani*. These findings give us a hope that the *de novo* pyrimidine pathway can be targeted for therapeutic interventions

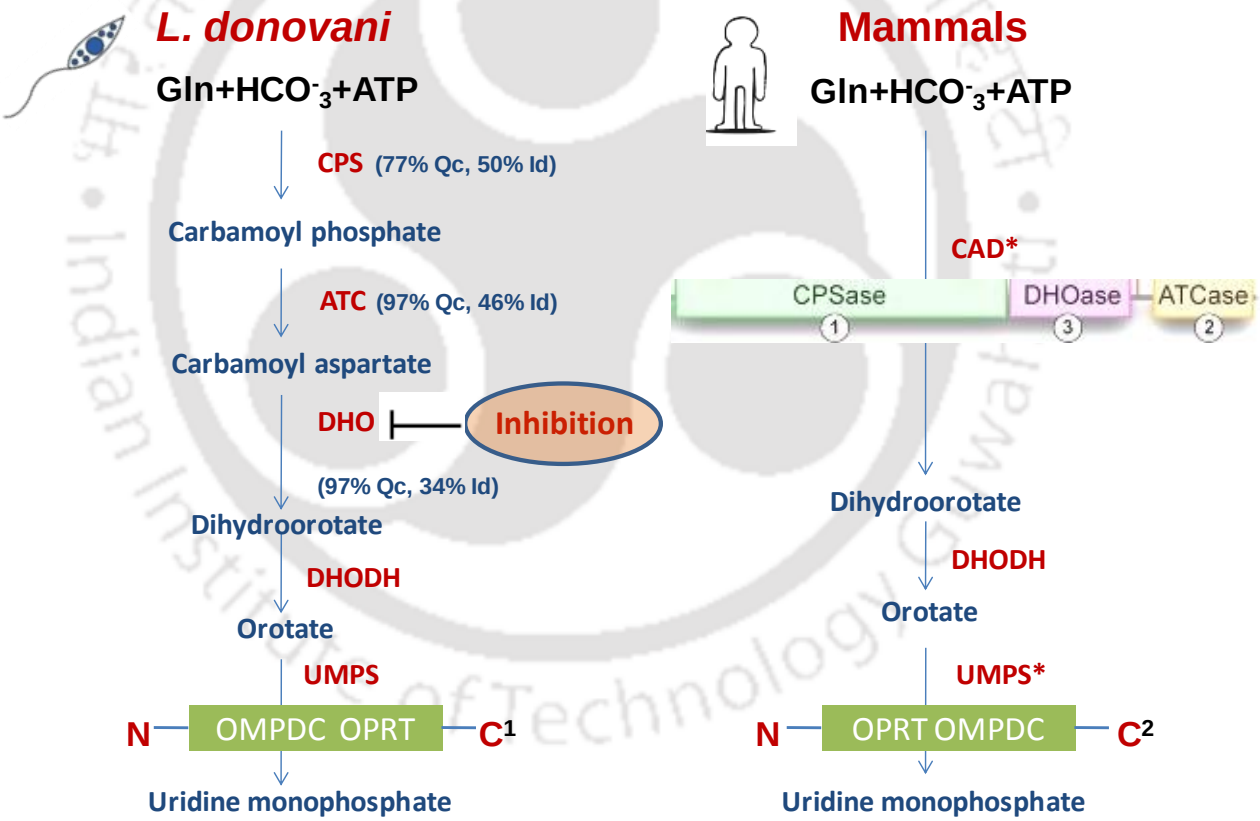

Figure 1.6: A comparative analysis of *de novo* pyrimidine biosynthesis pathway in *Leishmania donovani* and mammalian cells. All the steps of the pyrimidine biosynthesis pathway, except the one catalyzed by dihydroorotate dehydrogenase (DHODH), differ between the parasitic and mammalian pathway.

1.20 Dihydroorotase

Dihydroorotase (DHOase; E.C. 3.5.2.3) a metal dependent amidohydrolase catalyzes the third step in the *de novo* pyrimidine biosynthesis pathway. It is a zinc containing metalloenzyme that catalyzes the reversible interconversion between N-carbamoyl aspartate (CA-asp) and dihydroorotate (DHO). In *L. donovani* DHOase works as a monofunctional enzyme while in the host counterpart it is a part of a multifunctional enzyme complex. Phylogenetically DHOases have been classified into two types: Type I, high molecular weight (~45 kDa) found in eukaryotes and Type II, low molecular weight (~38 kDa) found in bacteria and fungi. *Leishmania donovani* DHOase falls in Type I category as its molecular weight is 44 kDa. High similarity (40%) is observed between the same types of DHOases while a lower degree (20%) of similarity is observed between different groups of DHOases. A further subdivision of Type I DHOases is done into Type IA, Type IB and Type IC. Type IA belongs to monofunctional enzyme such as *Ld* DHOase. Type IB enzymes need to be associated with one or more enzymes noncovalently to be functional for eg. DHOase from *Aquifex aeolicus* Type IC belongs to those DHOases which need to be covalently linked to atleast one or more enzymes to be functional for eg: CAD complex of mammalian cells.

1.21 Structure of *E. coli* dihydroorotase

Dihydroorotase is a zinc containing metalloenzyme belonging to the amidohydrolase superfamily. Enzymes belonging to amidohydrolase superfamily are marked by the presence of a $(\beta/\alpha)_8$ TIM barrel fold. Many DHOase exhibit a binuclear metal center (*E. coli* DHOase) while many other have a single zinc containing site (*A. aeolicus* DHOase). Crystal structures of DHOases from various species have revealed that the enzyme exhibits polymorphism at structural level and this variability can be exploited for the design of specific inhibitors which can be further evaluated for antiparasitic potentials. Dihydroorotase from *E. coli* has been crystallized and its structural determinants have been well defined thus the discussion will rely on the structural parameters of *E. coli* DHOase because a lack of crystal structure for *Ld* DHOase. However, wherever necessary the modeled structure of *Ld*DHOase would aid in the structural comparisons. *Escherichia coli* dihydroorotase was crystallized in the presence of its substrate N-carbamoyl aspartate. *Escherichia coli* dihydroorotase turns out to be a homodimer. The respective monomers are designated as subunit A and subunit B, where

each subunit contains an active site. In the crystal structure of *E. coli* DHOase DHO (product) was bound to subunit A while CA-asp (substrate) was bound to subunit B. Both the subunits exhibited same structure except a tetrapeptide (residues 109-112) orientation which lies in the loop region. The tetrapeptide containing Thr-109 and Thr-110 in subunit B leans towards the active site to form hydrogen bonds with substrate CA-asp. This conformation of the tetrapetide has been termed as loop in conformation. While the tetrapeptide in the subunit A formed a part of protein structure and did not form any hydrogen bonds with DHO in the active site thus displaying the loop out conformation. The loop in conformation is stabilized by hydrogen bonding with CA-asp while the loop out conformation is is stabilized by hydrogen bonding with neighbouring atoms of the protein. The loop regions are further stabilized by intra chain hydrogen bondings. The loop (residues 105-115) is a flexible region of the *E. coli* DHOase which can adopt loop in and out conformations *(Lee et al., 2007).* Both the subunits are bound by two zinc atoms each. The two zincs in the subunit B are bridged by the carboxylated lysine (Lys 102) as well by the carboxyl group of CA-asp. In the absence of substrate the two zinc atoms in subunit B are bridged by carboxylated lysine from the interior side and hydroxyl group of solvent from exterior side. The bridging hydroxide acts as a nucleophile in the hydrolysis of dihydroorotate and as leaving water molecule in the cyclization of dihydroorotate. The zinc atoms in the subunit A are bridged by the hydroxyl of the solvent. The active site of dihydroorotase usually contains four histidine, aspartate and carboxylated lysine residues. The two subunits of dihydroorotase in *E. coli* are mainly stabilized by salt bridges and hydrophobic interactions. Salt bridges formed by aspartate and arginine residues maintain the subunit interactions in *E. coli* DHOase. While hydrophobic interactions mediated by histidine, arginine and glutamic acid maintain the subunit interactions *(Robles Lopez et al., 2006).* In many other DHOases like *Bacillus anthracis* DHOase the two zinc atoms are bridged by carboxylated aspartate residue instead of lysine residue *(Mehboob et al., 2010).* Two highly specific inhibitors HDDP (2-Oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylic acid) and FOA were designed and tested against hamster DHOase. HDDP is a transition state analogue and a competitive inhibitor of DHOase. *E. coli* DHOase was also co-crystallized in the presence of HDDP.

1.22 Reaction mechanism of dihydroorotase

TIM barrel is a hallmark of amidohydrolase enzymes and thus the *Ec*DHOase structures as well as other DHOases are decorated with 8 parallel β-sheets connected by eight α-helices forming a TIM barrel. The TIM barrel structure is also evident in the modeled predicted structure of *Ld*DHOase as described in the results section. The loops connecting the C terminal of β-sheets to N terminal of α-helices form a cavity which is bound to zinc and forms the active site of the dihydroorotase (see results section). *Escherichia coli* DHOase has two zinc atoms bound to each subunit. The two zincs in both the subunits are bridged by a water molecule in the ligand free state. Both DHO and CA-asp interacted with the active site residues where CA-asp interacted with loop residues also. The mechanism behind the loop movement was explained on the basis of the conversion of CA-asp to DHO. Conversion of a carboxylate group of CA-asp to a carbonyl group of DHO prevents DHO to form a bridge between zinc atoms which otherwise was formed by CA-asp, thus the inability to form the bridge between two zinc atoms pushes out the DHO and thus the loop out conformation is established. The active site residues (Arg25, Asn55, Thr125) are well conserved in *Ld*DHO. The loop residues (residues 105-115) and zinc bridging residue (Lys117) which forms a bridge between two zincs are conserved in *Ld*DHOase. Also the zinc interacting residues (His21, His23 and Lys118) are found to be conserved in *Ld*DHO *(Lee et al., 2005)*.

1.23 *Aquifex aeolicus* dihydroorotase

Hamster DHOase is also a dimer when separated from its CAD complex. While monomeric DHOases have been reported for many parasitic species like *Crithidia fasciculata* and *Plasmodium berghei, Aquifex aeolicus* DHOase which is a Type I enzyme is reported to be a dodecamer having six DHOase and six ATCase subunits *(Edwards et al., 2013)*. The ATCase DHOase complex in *A. aeolicus* maintains a stoichiometry so that equal numbers of enzyme units are present. The complex in *A. aeolicus* is arranged in a manner such that the CA-asp synthesized by ATCase is channeled in the DHOase. ATCase and DHOase in the enzyme complex in *A. aeolicus* act in a coordinated manner such that when ATCase is inhibited by PALA (N-phosphonacetyl-L-aspartate) then the activity of distal DHOase is also affected. The loop region of AaDHOase has an interesting function as it swings back and forth between ATCase active site and DHOase and affects the catalytic activity of ATCase as well.

In solution AaDHOase existed as a monomer with single zinc bound to the active site. It gains its activity only when it associates with ATCase. *Staphylococcus aureus* also has single zinc in the active site of DHOase. DHOases from *B. anthracis (Mehboob et al., 2010)* and *T. thermophillus* possess two zinc atoms in the active site.

1.24 Structure and reaction mechanism of mammalian dihydroorotase

Mammalian DHOase which forms a domain of the multifunctional CAD complex was bound to two zinc atoms at its active site which were bridged by a carboxylated lysine residue. A third zinc atom was also found which coordinated with a rare histidinate ion. A flexible loop contacting the zinc atoms was also found at the active site. All the structural parameters including the TIM barrel were found to be conserved in human dihydroorotase domain of CAD complex. The flexible loop region of human DHOase oscillates between the open and closed conformation in an otherwise rigid structure. The mammalian DHOase is made up of two domains: the catalyticaly active β barrel domain and smaller another domain which is made up by the association of N-terminal and C-terminal residues. The coordination geometry of the two active site zincs is different in the Human DHOase *(Grande-García et al., 2014)*. The Zn-α is coordinated in a trigonal bypyramidal coordination while Zn-β is coordinated in a distorted tetrahedral geometry. The Zn-γ exhibited a tetrahedral geometry with binding in the β barrel. The β carboxylate group of CA-asp replaces the bridging water. Here the hydrolytic water molecule acts as a nucleophile. The negatively charged carboxylate group of CA-asp is neutralized by two Zn atoms and the C4 of CA-asp is rendered susceptible to nucleophilic attack by the deprotonated N3 of CA-asp. In this way the carbonyl-oxygen bond in the substrate is polarized to make the amide bond more electrophilic. Thus the catalytic activity requires the unprotonation of a single group. The interaction of T1562, a residue of the loop region with the β carboxylate group of the CA-asp further increases the electrophilicity of the C4 of CA-asp favoring the formation of dihydroorotate. Human DHOase favored the loop out conformation when bound to the inhibitor FOA. Mutations in the key loop residues T1562 and F1563 of human DHOase abrogated the catalytic activity indicating their key role in the catalysis; similarly mutations in the zinc coordinating residues impacted the activity of the enzyme. These all findings corroborated with those found for EcDHOase further strengthening the universality of the

reaction mechanism conserved in the DHOases. Human DHOase existed in the form of a homodimer in solution *(Grande-García et al., 2014; Hermoso, 2014)*. Strangely human DHOase is active in isolated form whereas AeDHOase is not as both DHOases are known to form complexes. The directionality of dihydroorotase is dependent on pH as the cyclization of CA-asp is favored in the slightly acidic pH while the hydrolysis of DHO is favored in slightly alkaline pH.

1.25 Inhibitors of *de novo* pyrimidine biosynthesis pathway

Inhibition of *de novo* pathway would lead to the depletion of nucleotides which would further cause DNA strand breaks which will lead to cell death via apoptosis. Thus the *de novo* pathway has been targeted in various modules including cancer and parasitic diseases. Condensation of carbamyl phosphate with aspartate to yield carbamyl aspartate mediated by ATCase is blocked by N-(phosphonoacetyl)-L-aspartic acid (PALA). PALA is analogous to both substrates in structure thus it is a bisubstrate analog *(Collins and Stark, 1971)*. PALA has been shown to be effective in eradicating solid tumors in mice. Dihydroorotase on the other hand has been targeted by dihydropyrimidine analogs like HDDP (2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate), TDHO (6-L-Thiodihydroorotate) and MMDHO. A second generation inhibitor MOAC which resembles the tetrahedral transition state has also been synthesized *(Manthey et al., 1998)*. Several boronic acid analogs which resemble carbamoyl aspartate have also shown promising inhibition with dihydroorotase. Oxidation of dihydroorotate by dihydroorotate dehydrogenase leads to the formation of orotate. The electrons thus released are accepted by ubiquinone in the inner mitochondrial membrane and enter the electron transport chain. Thus ubiquinone based analogs have been used to inhibit this step of the pyrimidine pathway. Several ubiquinine analogs like 1, 4 napthaquinone, lapachol and dicholoroallyl lawsone (chemical derivative of lapachol) are potent inhibitors of dihydroorotate dehydrogenase. Brequinar (fluorinated carboxyquinoline derivative) inhibits dihydroorotate dehydrogenase in a noncompetitive manner and has been used as an anticancer agent *(Chen et al., 1986)*. Another ubiquinone analog leflunomide which is an anti-inflammatory agent also inhibits dihydroorotate dehydrogenase *(Elder et al., 1997)*. Atavoquone an antimalarial drug inhibits dihydroorotate dehydrogenase in an indirect manner by forming covalent adduct with complex III of electron transport chain in inner

mitochondrial membrane *(Fry and Pudney, 1992)*. The final step of the pyrimidine biosynthesis pathway catalyzed by ODCase is inhibited by numerous inhibitors. Among them pyrazofurin (antimalarial) and nucleoside 5'monophosphate derivative of barbiturate (BMP) are potent inhibitors of ODCase *(Scott et al., 1986)*. Another analog 6-azauracil which also possesses anticancer activity also inhibits ODCase *(Levine et al., 1980)*.

In another report a small molecular weight compound A3 identified through a highthroughput screen was shown to restrict the growth of influenza virus in a dihydrooortate dehydrogenase dependent manner. The A3 compound acts by inhibiting the pyrimidine biosynthesis, thus starving the virus *(Hoffman et al., 2011)*. Flavonols and flavones are types of flavonoids commonly found in plants such *Ginkgo biloba*, *Tilia spp*, *Equisetum spp* and are responsible for the color and flavor of fruits. Among the flavonols kaempferol, myricetin and quercitin are aromatic compounds having a heterocyclic pyran-4-one ring and are known to be potent inhibitors of amidohydrolases. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) which has a diphenylpropane structure has been a reported inhibitor of *Staphylococcus aureus* dihydroorotase and *Klebsiella pneumoniae* allantoinase *(Peng and Huang, 2014)*. Protection against various types of cancers has also been observed by the application of kaempferol *(Dang et al., 2015)*. Kamepferol is well known for its antioxidative properties and chemotherapeutic potential.

1.26 L-Asparaginase

L-asparaginases (EC 3.5.1.1) are amidohydrolases that bring about the formation of aspartic acid by hydrolyzing the amide bond of asparagine, and they are widely distributed in all three domains of life. The release of ammonia can be measured by Nessler test. Asparaginases play a pivotal role in aspartate metabolism which also affects Krebs cycle and pyrimidine metabolism as it provides precursors like carbamoyl aspartate and oxaloacetate for both of these pathways respectively. Two isozymes of L-asparaginase are usually found in the cells, Type I is predominantly localized in the cytoplasm and has lower affinity to the substrate while the Type II isozyme is localized in periplasm of *E. coli* i.e secreted out and has higher affinity to the substrate. Owing to the higher affinity to asparagine the Type II asparaginases have found their remarkable application in the treatment of childhood acute lymphoblastic leukemia (ALL) as the tumor cells are deficient in the asparagine synthetase enzyme thus are

unable to synthesize asparagine and thus rely on the extracellular supply of asparagine which is cut off by the application of asparaginase leading to starvation of tumor cells. Asparagine is not an essential amino acid but is needed by tumor cells for their survival. *E. coli* Type II enzymes are sold in the trade name of Elspar and Oncospar (conjugated with polyethylene glycol). *Erwinia chrysanthemi* asparaginase is sold in the trade name of Erwinase. Type II asparaginases have been widely characterized as compared to the Type I isozymes. Various Type II asparaginases from *Erwinia chrysanthemi, Acinetobacter glutaminasificans* etc. have been charcterized and their crystal structures are available. Both isozymes of asparaginase are also produced by *S. cerevisiae* in which the Type II isozyme is secreted out in response to nitrogen starvation *(Dunlop et al., 1978)*. Asparaginase in the form of drug formulations is administered to the patient's suffering from ALL. These include the native L-asparaginase from *E. coli*, L-asparaginase in PEGylated form (PEG: polyethylene glycol) and also *Erwinia chrysanthemi* asparaginase. PEGylated form of asparaginase has reduced sideeffects. Asparaginases exert their toxic effects as they have intrinsic glutaminase activity which causes reduced L-glutamine intake by the cells of liver and pancreas. Asparaginases are constitutively produced in various microbes. A study has reported the involvement of asparagine in the autophagy pathway of *L. donovani (Stephanie et al., 2006)*. In another study it has been pointed out that the acidic environment inside the host cell was weakened by the *M. tuberculosis* asparaginase to confer its survival *(Gouzy et al., 2014)*. The active site residues are conserved in *L. donovani* asparaginase while two ORFs for asparaginase are present in *L. donovani* genome, one at chromosome 15 and another on chromosome 36. Coding sequence of chromosome 15 relates with the type I asparaginase and has been annotated as cytoplasmic asparaginase like protein while the coding sequence of chromosome 36 also relates to type I asparaginase which is annotated as asparaginase like proten in the GeneDB database.

1.27 Structure and reaction mechanism of asparaginase

Most of the Type II asparaginases are homotetramer where each monomer contains the active sites. Crystal structure of first Type I asparaginase was solved in *Pyrococcus horikoshii* which is a hyperthermophilic archaeon. Asparaginases are made up of two α/β domains which are joined by a linker. A large domain is formed by N terminal and a small domain is

formed by C terminal of the protein. The N terminal domain is mainly made up of 12 β strands and 4 α helices which together form a flavodoxin like motif. While the small C terminal domain is formed by 5 α helices and 4 stranded parallel β strands. Type I asparaginase from *P. horikoshii* is a homodimer. The active site is formed by a flexible loop. For long it was speculated that human beings lacked asparaginase but recent reports has confirmed the presence of a Type III asparaginase which are very diverse from the TypeI and Type II asparaginases in their catalytic mechanisms. Human type III asparaginase is an Ntn (N-terminal nucleophile) hydrolase which requires autocleavage to gain activity; however the K_m for asparagine is very high making this enzyme inefficient in the catalysis of asparagine. Human genome codes for three enzymes which show asparaginase activity: L-asparaginase (hASRGL1/ALP/CRASH), lysophospholipase and aspartylglucosaminidase (hAGA). Human L-asparaginase can also hydrolyze isoaspartyl peptide linkages and has homology with *E. coli* Type III asparaginase. Lysophospholipase, a 60 kDa enzyme can catalyze lysophospholipids and its N terminal domain is homologus to *E. coli* Type II enzyme, while aspartylglucosaminidase is localized in the lysosomes and breaks the bond between asparagine and carbohydrates in cell surface glycoproteins.

Human L-asparaginase is Ntn hydrolase which brings about their autocatalysis to generate α and β chains which provide the catalytic activity to the enzyme. However the rate of self cleavage is very slow in case of human asparaginase, while the *E. coli* Type III asparaginase undergoes rapid cleavage to yeild active fragments. Active sites of all asparaginases are composed of a catalytic triad comprising serine, histidine and aspartic acid where serine acts as a nucleophile, histidine as a base and aspartic acid as an acid. The catalytic triad plays a key role in the reaction mechanism of asparaginases. In the first step of the reaction the nucleophile is activated by the adjacent base which then attacks the carbon atom of the amide substrate. This leads to the formation of an enzyme acyl intermediate state which develops negative charge on the oxygen atom of the amide group. The negative charge on the oxygen is counter balanced by hydrogen bonding from adjacent groups leading to the formation of an oxyanion hole. In the second step of the reaction water acting as a nucleophile attacks the carbon atom of the ester following the similar series of steps including the transition state *(Michalska and Jaskolski., 2006).*

Type I asparaginase (AnsA) from *E. coli* are reported to be dimers. Constitutive expression of Type I asparaginase is found in *E. coli* while Type II asparaginase (AnsB) is only produced in anaerobic conditions and nutrient starvations. Expression of Type II asparaginase is monitored by cAMP and oxygen sensing mechanism regulator of fumarate and nitrate reduction (FNR) protein. Type II enzymes which are primarily secreted out are designed by nature to scavenge the available nitrogen which is very less in external environment, thus they have evolved to have high affinity to their substrates (low K_m). Highly conserved two α/β domains connected by a linker are also present in *E. coli* asparaginase. A larger N terminal domain and a smaller C terminal domain are present. N terminal domains are mainly responsible for the oligomerization of *E. coli* Asparaginase. An unexpected allosteric mode of regulation of *E. coli* asparaginase by asparagine was observed by analyzing the crystal structure which revealed the bound asparagine at the N terminal domain other then their active sites. The allosteric mode of action by *E. coli* AnsA was also supported by the kinetic data which showed that the enzyme activity followed Hill equation. Only the intracellular asparaginase in *E. coli* exhibits sigmoidal substrate activity dependence i.e cooperativity while the extracellular enzyme does not show cooperativity *(Yun et al., 2007)*. In a similar way, *S. cerevisae* intracellular asparaginase also exhibits cooperativity while the extracellular enzyme is devoid of cooperativity. PhAnsA although does not exhibit cooperative behavior. *Escherichia coli* crystal structure reveals a ping pong nucleophilic attack on the substrate leading to amidohydrolysis. Two threonines act as a nucleophile in the catalytic activity of the *E. coli* asparaginase *(Yun et al., 2007)*. First threonine is located in the loop region which mediates the first nuclephilic attack on substrate while the second attacking threonine is a part of a more rigid structure. However a third category of asparaginase (Type III) is also present in *E. coli* which resembles the structure of glycosylasparaginases. The Type III *E. coli* asparaginase is actually isoaspartyl peptidase with Ntn hydrolase like activity and is a heterotetramer. The EcaIII is encoded by an *ybiK* gene. In EcaII also theronine acts as a nucleophile.

1.28 Role of asparaginase in pathogenesis

What role does asparaginase plays at host pathogen interface is interesting to know. Whether it aids in its survival or not are the key questions that need to be addressed. One striking

reveletion has been made in *Mycobacterium tuberculosis* which is an intracellular pathogen like *L. donovani* and resides in the acidic phagosome of mammalian cells. According to the study the secreted asparaginase of *M. tuberculosis* converts the asparagine into aspartic acid and ammonia in the phagosome, the released ammonia thus neutralises the acidic compartment and aids in the survival of *M. tuberculosis.* Asparaginase mutants of *M. tuberculosis* were found to be attenuated in growth with lower infectivity. The study has provided enough evidence that asparagine serves as a key nutrient in the survival of *M.* tuberculosis.Taking clue from *M. tuberculosis* based studies the same can be reflected back in the *L. donovani* mediated infection *(Gouzy et al., 2014). Leishmania donovani* delays the phagosome maturation and fusion with lysosome as one of its surviving virulence strategies. Where the asparaginase fits in is the intriguing question that needs to be addressed. Also the presence of two isoenzymes of asparaginase in *L. donovani* makes this assumable that asparaginase may play a key role in the infectivity. In a recently reported study Type II L-asparaginase has been shown to be a key player in the infectivity of *Salmonella typhimurium (McLaughlin et al., 2016). Salmonella typhimurium* mutants lacking Type I and II asparaginases were poor in colonizing the host tissue. Type II asparaginase produced by *S. typhimurium* also hampered the T cell mediated response and decreased cytokine production. Many human pathogens like *Helicobacter pylori*, *Campylobacter jejuni* and *Francisella tularensis* require asparagine catabolism for maintaining their infecivity and survival inside the host cell.

Aspartate metabolism plays a key role in maintaining the proper nitrogen pool of a cell thus it becomes obvious that the parasites may utilize this machinery for their own benefit. A study done on *Mycobacterium tuberculosis* reflects this notion as when the intracellular pathogen was starved with nitrogen the key upregulated genes also include those of aspartate metabolism pathway. Although ammonium has been suggested as a preferred nitrogen source for bacteria but growing evidence suggests that asparagine may be the preferred nitrogen source *(Williams et al., 2015).* Another extracellular pathogen Group A *Staphylococcus* (GAS) utilizes the host asparagine produced as a result of asparagine synthetase which is upregulated by the induction of host ER stress caused by streptolysin toxin of bacteria. Asparaginase II has been shown to be effective in reducing the infectivity of GAS *(Baruch et al., 2014).* Although several inhibitors of asparaginase has been reported in the literature,

among them only ASA (aspartic β semialdehyde) and DONV (5-diazo-4-oxo-L-norvaline) prove to be the potent inhibitors of asparaginase. ASA has been identified as a reversible inhibitor of *E. coli* asparaginase *(Westerik and Wolfenden, 1974)*. DONV is an L-asparagine analog which binds to the catalytic site of the enzyme in an irreversible manner *(Jackson and Handschumacher, 1970)*. DONV displayed stronger inhibition against *E. carotovora* asparaginase then to *E. coli* asparaginase.

1.29 Significance of the current work

Two key pathways, *de novo* pyrimidine biosynthesis pathway and aspartate metabolic pathway, have been investigated in the current work using a combination of biochemical and genetic approaches in *Leishmania donovani* parasite. The presented work would aid in the better understanding of the *de novo* pyrimidine biosynthesis pathway in the *L. donovani* parasite. Dihydroorotase enzyme inhibition approach would lead us to better understand the interplay between the *de novo* and salvage pyrimidine pathway in the *L. donovani* parasite. Various structural differences between third enzyme of *de novo* pyrimidine biosynthesis pathway of *L. donovani*, dihydroorotase, and its mammalian counterpart have been highlighted. These structural insights would be useful in the development of rational and specific inhibitors of the pyrimidine pathway. The two inhibitors, kaempferol and biotin sulfone identified in the current investigation would be helpful in probing and understanding the pathway, further these inhibitors could be used as parent or lead compounds for the development of therapeutic interventions for visceral leishmaniasis **(Figure 1.7)**.

Furthermore, a key enzyme of the aspartate metabolic pathway: *Leishmania donovani* asparaginase provides precursors to the *de novo* pyrimidine pathway and is presumed to be absent in the mammalian counterpart, thus its subcellular localization was determined. The experimentally predicted localization pattern of the asparaginase variants in the *L. donovani* parasite would aid in understanding the role played by the asparaginase variants in the growth and infectivity of the parasite. An understanding of the pyrimidine and aspartate metabolic pathways would be helpful in the development of combinatorial therapy targeting two enzymes thus enhancing the potency.

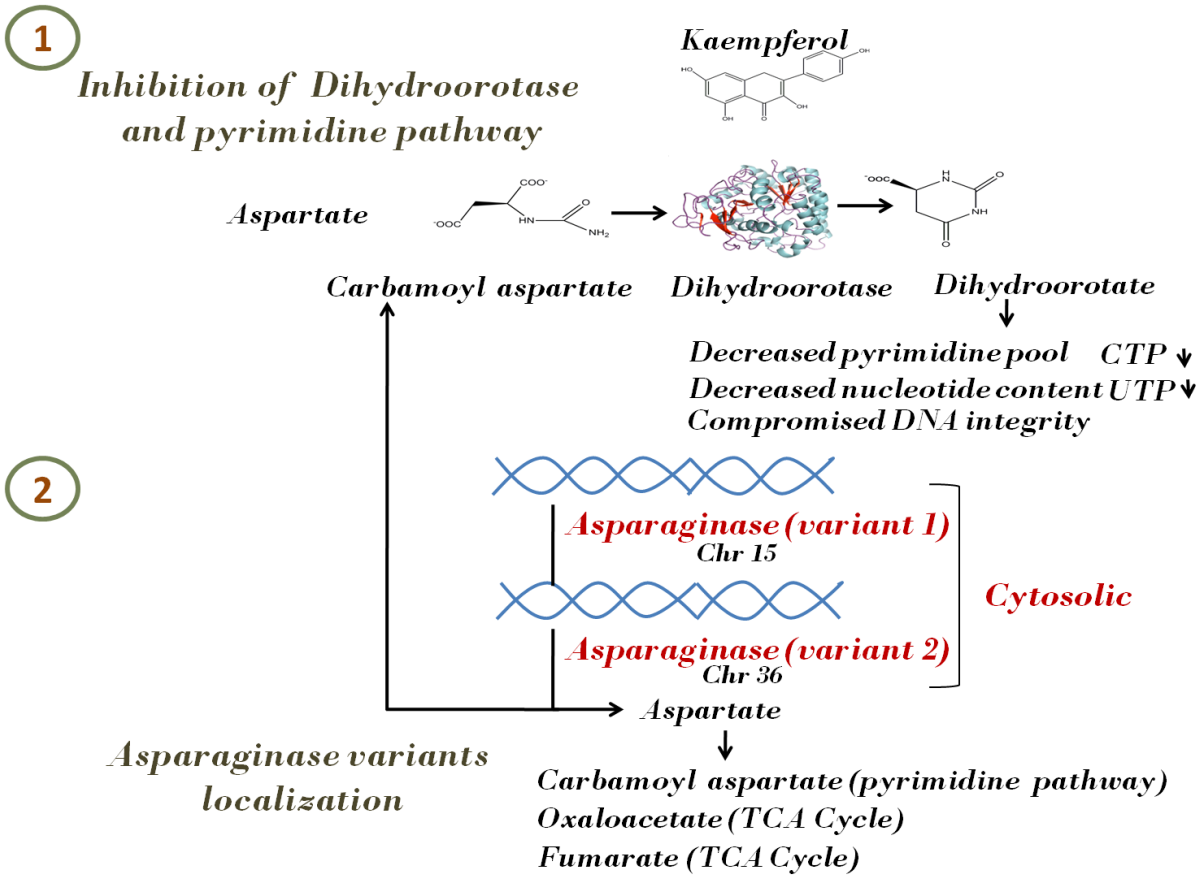

Figure 1.7. An outline of the work. **(1)** Dihydroorotase inhibition by kaempferol. Aspartate and pyrimidine metabolic pathways were investigated by localization and small molecule inhibition approaches. Both genetic and biochemical approaches were employed to validate the bonafide functions of the asparaginase as well as dihydroorotase enzymes, also these approaches were employed to investigate the aspartate and pyrimidine biosynthesis pathways and their overall role in the survival of the *Leishmania donovani* parasite. **(2)** Asparaginase localization in *Leishmania donovani*.

CHAPTER 2

Cloning, expression and biochemical characterization of *Leishmania donovani* dihydroorotase*

ABSTRACT

Leishmania donovani dihydroorotase, the third enzyme of the *de novo* pyrimidine biosynthesis pathway was cloned and expressed in *E. coli* BL21(DE3) and purified to homogeneity with an observed band size of 48 kDa on SDS-PAGE. The enzyme was biochemically characterized in both directions since it catalyzes a reversible reaction. Kinetic characterization for the forward reaction gave a K_m of 28.1± 6.5 μM and a V_{max} of 1.2 ± 0.06 μMs^{-1}, while for the reverse reaction the estimated K_m was 220.75 ± 15.3 μM and a V_{max} of 0.404 ± 0.012 μMs^{-1}. Owing to the dwelling of digenetic parasite *L. donovani* in two differing pH (alkaline and acidic), the pH optima was determined to be 6.0 for forward reaction and 8.0 for reverse reaction. Dihydroorotase existed in the form of a tetramer as revealed by size exclusion chromatography. In conclusion, the dihydroorotase favored forward reaction over reverse reaction while the forward reaction was favored in a slightly acidic pH and reverse reaction was favored in a slightly alkaline pH.

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2.1 Introduction

Visceral leishmaniasis (VL) is a neglected tropical disease prevalent in 98 countries across the globe with 350 million people at risk of infection. The disease is endemic in India and caused by a vector borne unicellular protozoan parasite *Leishmania donovani (Singh et al., 2006; Alvar et al., 2012; Saudagar et al., 2013). Leishmania donovani* is transmitted through the bite of a female sandfly *Phlebotomus* (in the old world) and *Lutzomyia* (in the new world) to the vertebrate host *(Volf et al., 2008).* Few treatment regimens have been developed over the years to treat this fatal disease. However, among the available treatment options, pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) have been used as a first line of treatment for more than 80 years. The other available drugs like amphotericin B, paromomycin and the only available oral drug, miltefosine, are current preference for treatment options *(García-Hernández et al., 2015).* In addition to toxicity issues related with these available drugs, emergence of resistant strains of *Leishmania* in Indian subcontinent is the greatest concern in the recent times *(Ashutosh et al., 2007).*

Pyrimidines are vital components of a cell as they are not only required as precursors for DNA and RNA synthesis *(Jones, 1980)* but they also serve as sources of energy in the form of UTP (glucose and galactose activation) and CTP (lipid metabolism) *(Fairbanks et al., 1995).* Also uridines have been reported to act via extracellular receptors, thus regulating the cellular physiology *(Connolly and Duley, 1999).* Pyrimidines are synthesized by a series of six sequential steps catalyzed by six enzymes *(Jones, 1980).* Dihydroorotase (DHOase) (EC 3.5.2.3), a zinc metalloenzyme of the amidohydrolase superfamily, is the third enzyme of the pathway catalyzing the reversible cyclization of N-carbamyl aspartate to dihydroorotate. The first cyclic product of pyrimidine pathway, dihydroorotate, is synthesized by dihydroorotase enzyme. Dihydroorotases of the amidohydrolase superfamily have been classified into two classes *(Fields et al., 1999)*: Type I, which are multifunctional enzymes of high molecular weight (~45 kDa) like mammalian DHOase *(Grande-García et al., 2014: Ruiz-Ramos et al., 2015),* while type II are monofunctional and small (~38 kDa) found mostly in bacteria and fungi *(Grande-García et al., 2014).* This the first hand report describing the biochemical characterization of *L. donovani* dihydroorotase with its oligomeric status.

2.2 Materials and methods

2.2.1 Materials

Leishmania donovani strain BHU 1081 was a kind donation from Prof. Shyam Sundar, Banaras Hindu University, India. The culture conditions for *L. donovani* are well established in our laboratory *(Saudagar et al., 2013, Das et al., 2015)*. In brief, the *L. donovani* was grown in M199 medium supplemented with 15% heat inactivated fetal bovine serum (FBS), 100Uml^{-1} and 100 µgml^{-1} streptomycin. For inhibition and gene expression based experiments M199 media was supplemented with 15% dialyzed heat inactivated fetal bovine serum. The DreamTaq Green DNA polymerase was purchased from Thermo scientific. All restriction enzymes were purchased from ILS (NEB). Isopropyl β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Merck. *N*-carbamoyl-L-aspartic acid (CA-asp) was ordered from MP Biomedicals and L-dihydroorotate (DHO) and kaempferol were ordered from Sigma Aldrich Chemicals. Biotin sulfone was purchased from Enamine. All other chemicals were of highest purity grade, procured from Sigma Aldrich or Merck.

2.2.2 Cloning of L. donovani dihydroorotase (LdDHOase)

Genomic DNA was isolated from *L. donovani* using Qiagen genomic DNA isolation kit. Forward primer 5'-CG*GGATCC*ATGTCCTCGGCCCCCATG-3' (underlined sequence is restriction site for *Bam*HI) and Reverse primer 5'-CCC*AAGCTT*TCACTCCTTTGCCAGGG-3' (underlined sequence is restriction site for *Hind*III) specific for *Ld*DHOase gene (Gene DB: LdBPK_160580.1) were used with *Bam*HI (NEB) and *Hind*III (NEB) sites incorporated into them. The gene was amplified by PCR in following conditions: initial denaturation at 95 °C, 5 minutes followed by 30 cycles of denaturation at 94°C, 30 sec, annealing 68°C, 30 sec, extension 72 °C, 30 sec and final extension at 72°C, 10 minutes. ~1218bp amplicon was observed on 1% agarose gel stained with ethidium bromide. The PCR product along with the cloning vector pET-28a(+) (Novagen) were subjected to restriction digestion by *Bam*HI and *Hind*III, and ligated to pET-28a(+) vector using T4 DNA ligase. Recombinant pET-28a(+) was transformed into *E.coli* DH5α cells and the clones were screened for the presence of DHOase gene by restriction

digestion, and final confirmation was done by sequencing using T7 forward and T7 reverse primers.

2.2.3 Expression and purification of L. donovani dihydroorotase

Recombinant pET28a-*Ld*DHOase vector was transformed into competent *E. coli* BL21 (DE3) cells for heterologous expression of protein by heat shock method **(Figure 2.1)**. Single colony was picked up and inoculated in 5mL Luria Broth overnight for primary culture. 1% primary culture was used to inoculate 500mL secondary culture for 2-3 hours. When the optical density of the culture reached 0.4-0.6 the culture was cooled down to 18°C and induced by adding 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) to it and kept for 14 hours in an incubator shaker maintained at 18°C and 180 rpm. After 14 hours the culture was cooled down on ice for around 30 minutes and pelleted down thereafter at 5000 rpm, 5 minutes at 4°C. The pellet was resuspended and incubated for few minutes in lysis buffer (20 mM Tris-HCl, 250 mM NaCl and 2.5% glycerol, pH 8.0). This was followed by sonication with a pulse of 6 sec on and 10 sec off cycle, 30% amplitude for 30 minutes. The bacterial lysate was clarified by centrifuging at 13,000 rpm for 30 minutes at 4°C to obtain the supernatant which was filtered using 0.45-µm membrane filter. The N-terminal His tagged protein was eluted out by using Nickel NTA metal affinity chromatography. The Ni NTA column was prepared by charging with 1M $NiSO_4$ and pre- equilibrating it with 10 mM imidazole prepared in binding buffer (20 mM Tris-HCl, 250 mM NaCl, pH 8.0). The membrane filtered supernatant was mixed with charged Ni NTA beads and kept on a rocker in cold room for about 1 hour for proper binding. The Ni NTA bound protein mixture was loaded on to the column and the protein was eluted by applying an imidazole gradient of 20 mM to 300 mM. The recombinant protein was finally eluted at 300 mM imidazole. The recombinant protein was then subjected to overnight dialysis in dialysis buffer (20 mM Tris-HCl, 250 mM NaCl, pH 8.0). Initially dialyzed in two liters of dialysis buffer and then transferred to 1 liter of buffer. Protein concentration was estimated by Bradford assay using BSA as the standards. Protein size was estimated by running in 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.4 Determination of oligomeric state

Size exclusion chromatography was performed to determine the oligomeric state of recombinant *Ld*DHOase. Briefly Sephacryl S-300 size exclusion column with a column volume of 70 mL was washed with 2 column volumes of filtered and degassed Milli-Q water followed by pre-equilibratation with 2 column volumes of 20 mM Tris buffer (pH 8.0). Briefly, purified recombinant *Ld*DHOase was concentrated using Amicon ultra centrifugal filter (Millipore Corporation). The column (Column Volume: 70 mL) was coupled with AKTA^{TM} FPLC system (GE Healthcare Life Sciences). The protein was eluted at a flow rate of 0.8 mL/min and fractions of 3 mL were collected in each tube. Various protein standards including alkaline phosphatase, bovine serum albumin (BSA) and lysozyme were also subjected to chromatography under same conditions and a standard curve was plotted between K_{av} and Log Mw (Where Kav = Ve-Vo/Vc-Vo; Ve: Elution volume, Vo: Void volume, Vc: Column volume). The eluted protein peak was compared to the protein standard and the oligomeric state was deduced out.

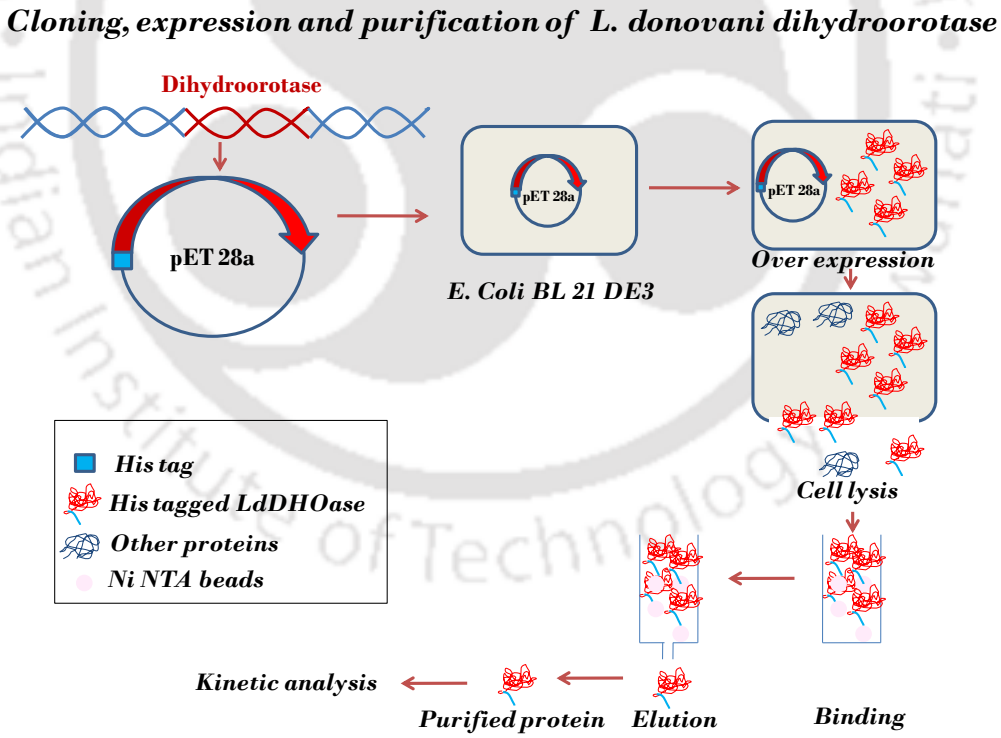


Figure 2.1. Schematic for cloning and expression of *L. donovani* dihydroorotase. Dihydroorotase gene was PCR amplified from the *Leishmania donovani* genomic DNA and cloned in pET28a expression vector which was further transformed in to *E. coli* BL 21(DE3) cells for the heterologus expression of recombinant his tagged dihydroorotase which was purified from the bacterial lysate using Ni NTA metal affinity chromatography.

2.2.5 Enzyme kinetic characterization

Enzyme activity for the purified recombinant DHOase was measured in the degradative (reverse) direction by monitoring the formation of *N*-carbamoyl-L-aspartic acid (CA-asp) at 230 nm which led to a decrease in absorbance due to ring cleavage of dihydroorotate (DHO). The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 8.0), 500 μM dihydroorotate, and the reaction was initiated by the addition of 0.6 μM of enzyme. All reactions were performed in triplicates and were carried out at 25°C for 10 minutes *(Truong et al., 2013).* Decrease in absorbance at 230 nm was measured on UV-Vis spectrophotometer (Agilent Spectrophotometer).

Kinetic parameters like K_m, V_{max} and k_{cat} were determined for the enzyme in both forward and reverse directions. Reactions in biosynthetic and in ring cleavage directions, both commence at different buffer pH as described in previous findings *(Christopherson and Jones, 1980: Taylor et al., 1976).* The enzyme concentration was optimized to obtain maximum linearity. Reaction for forward direction contained *N*-carbamoyl-L-aspartic acid in varying concentrations (100μM to 1000μM) in 50 mM KH_2PO_4 buffer (pH 6.0) with 0.6 μM of enzyme added into it and was incubated at 25°C for 10 minutes. Reverse reaction was carried out in 50 mM Tris buffer (pH 8.0) containing 0.6 μM of enzyme, and varying dihydroorotate concentrations (100 μM to 1000 μM). The reaction mixture was incubated at 25°C for 10 minutes and absorbances were measured at 230 nm. Data was fitted to Michaelis-Menten equation and double-reciprocal plot was obtained from which K_m and V_{max} were estimated. A pH range of 4.0-11.0 was employed to determine the pH optima of the purified recombinant *Ld*DHOase. Three buffers: 50 mM sodium acetate (pH 4.0-5.0), 50 mM KH_2PO_4 (pH 6.0-7.0) and 50 mM Tris buffer (pH 8.0-11.0) were used to obtain the desired pH range of 4.0-11.0. Substrates for the forward and reverse reactions were dissolved to a final concentration of 0.5 mM in respective buffers. 0.6 μM enzyme was added in the reaction mixture to initiate the reaction followed by incubation at 25°C for 10 minutes, and absorbance was measured at 230 nm. Appropriate substrate blanks were included in all the reactions. The pH optima were determined by plotting relative activity at different pH values *(Truong et al., 2013).*

2.3 Results

2.3.1 Cloning, expression and purification of L. donovani dihydroorotase

The open reading frame for *Ld*DHOase gene is 1218 bp long. The predicted molecular weight of protein is 43.9 kDa and a pI of 6.0. The pET28a-*Ld*DHOase construct was confirmed by double digestion, showing release of 1218 bp fragment on 1% agarose gel stained with EtBr **(Figure 2.2)**. The clone was further confirmed by sequencing using T7 promoter and T7 reverse primers. The pET28a-*Ld*DHOase construct was transformed into *E.coli* BL21 (DE3) cells and soluble fraction of protein was obtained at 0.5 mM IPTG concentration, 14 hrs incubation at 18°C. The recombinant protein was purified by Ni-NTA chromatography and purity was checked on 12 % SDS-PAGE **(Figure 2.3)**. The His-tagged recombinant protein showed a characteristic band of 48 kDa on SDS-PAGE as shown on **Figure 2.3C**. The purified recombinant *Ld*DHOase was subjected to size exclusion column. Standards including alkaline phosphatase, BSA and lysozyme were also applied to the column and eluted protein was compared with standards, it appears to be a tetramer **(Figure 2.4)**.

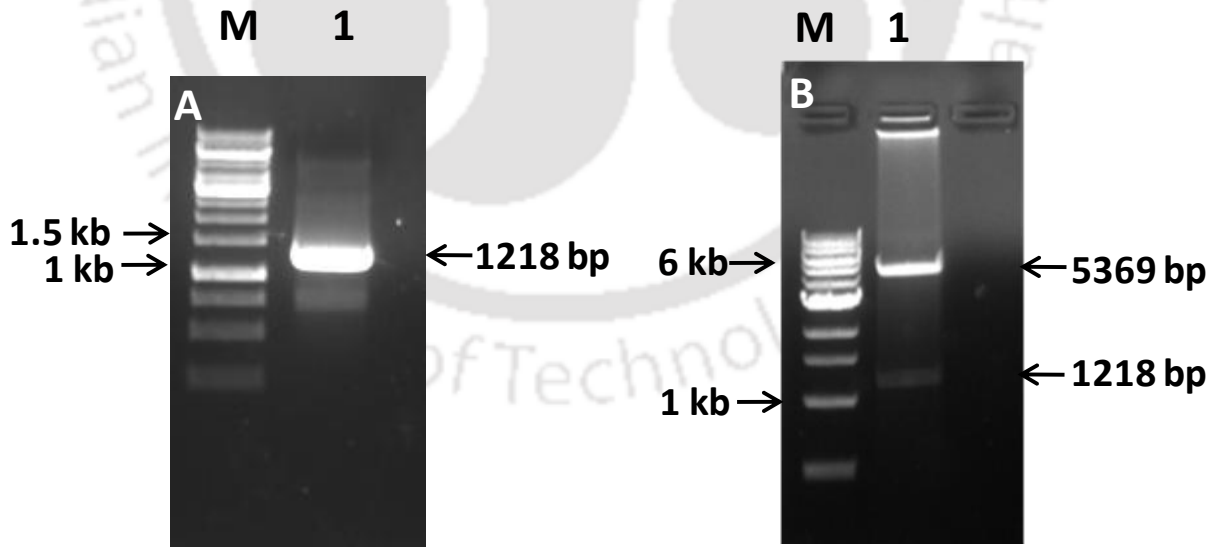


Figure 2.2. Cloning of dihydroorotase in pET 28a expression vector. **(A)** PCR amplification of dihydroorotase gene from *L. donovani* genomic DNA. Lane M: 1kb DNA ladder. Lane 1: PCR amplicon (1218 bp) **(B)** Restriction enzyme digestion of pET28a to release the dihydroorotase gene fragment. Lane M: 1kb DNA ladder. Lane 1: Clone confirmation by double digestion of pET 28a vector by *Bam*HI and *Hind*III and release of 1218 bp fragment. All gels were run on 1% agarose and stained with ethidium bromide.

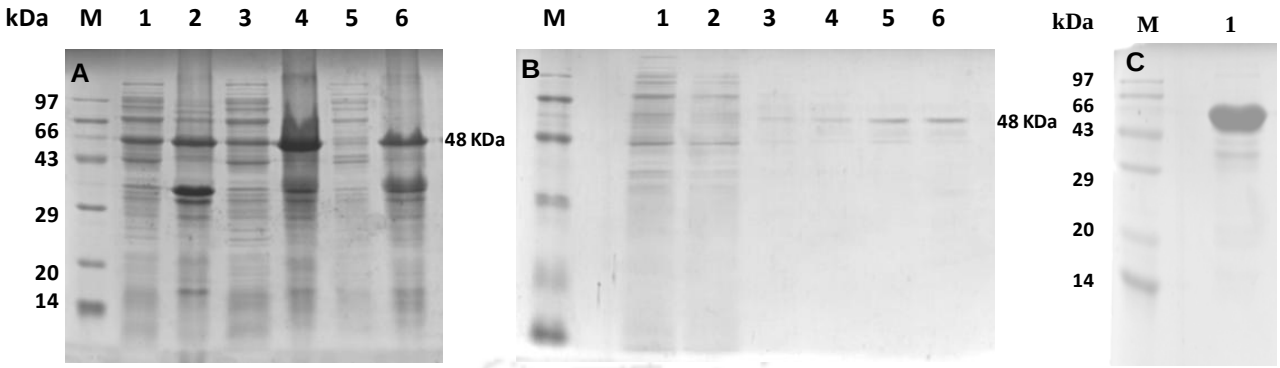


Figure 2.3. Expression and purification of *L. donovani* dihydroorotase run on SDS-PAGE and stained with Coomassie brilliant blue G 250 **(A)** Protein expression optimization at various temperatures at an induction of 0.5 mM IPTG. Lane M: Protein molecular weight marker. Lane 1: Induced (0.5mM IPTG) protein at 18°C (supernatant). Lane 2: Induced (0.5mM IPTG) protein at 25°C (supernatant). Lane 3:Induced (0.5mM IPTG) protein at 37°C (supernatant). Lane 4: Induced (0.5 mM IPTG) protein at 18°C (pellet). Lane 5: Induced (0.5 mM IPTG) protein at 25°C (pellet). Lane 6: Induced (0.5 mM IPTG) protein at 37°C (pellet). **(B)** Protein purification using Ni NTA column. Lane M: Protein molecular weight marker. Lane 1: Unbound protein fraction. Lane 2: 20 mM imidazole elute. Lane 3: 50 mm imidazole elute. Lane 4: 100 mM imidazole elute. Lane 5: 300 mM imidazole elute. Lane 6: Dialyzed 300 mM imidazole elute. **(C)** Protein purification from large scale culture. 48 kDa purified recombinant dihydroorotase.

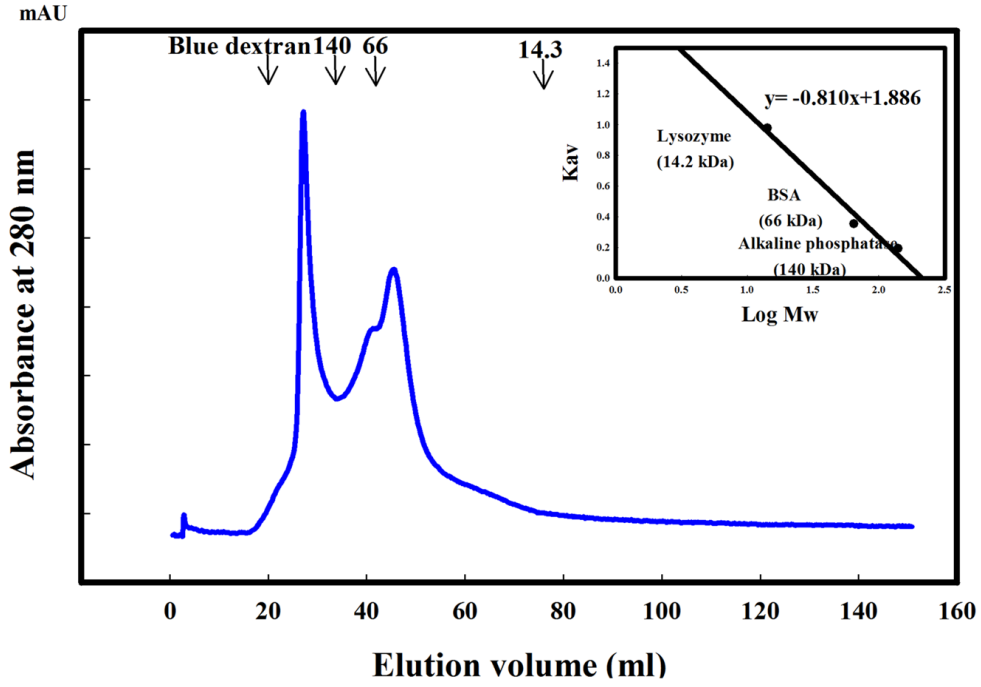


Figure 2.4. Size exclusion chromatography of *Ld* dihydroorotase with an expected tetramer state.Inset showing the protein standard curve plotted between Kav and Log Mw. The chromatogram displays two distinct peaks which are proposed to be tetramer and dimer respectively when compared with the protein standard curve. The dimer peaks surpasses the tetramer peak in terms of peak area which raises the possibility of existance of an equilibrium between dimers and tetramers of dihydroorotase.

2.3.2 Kinetic characterization of recombinant L. donovani dihydroorotase

*Ld*DHOase catalyzes a reversible reaction. Thus, the kinetic parameters were estimated for both forward and reverse reactions. For the forward reaction K_m (CA-asp) and V_{max} (CA-asp) were 28.1 ± 6.5 μM and 1.2 ± 0.06 μMs^{-1}, for the reverse reaction K_m (DHO) and V_{max} (DHO) were 220.7 ± 15.3 μM and 0.404 ± 0.012μMs^{-1} respectively **(Figure 2.5 and Table 2.1)**.

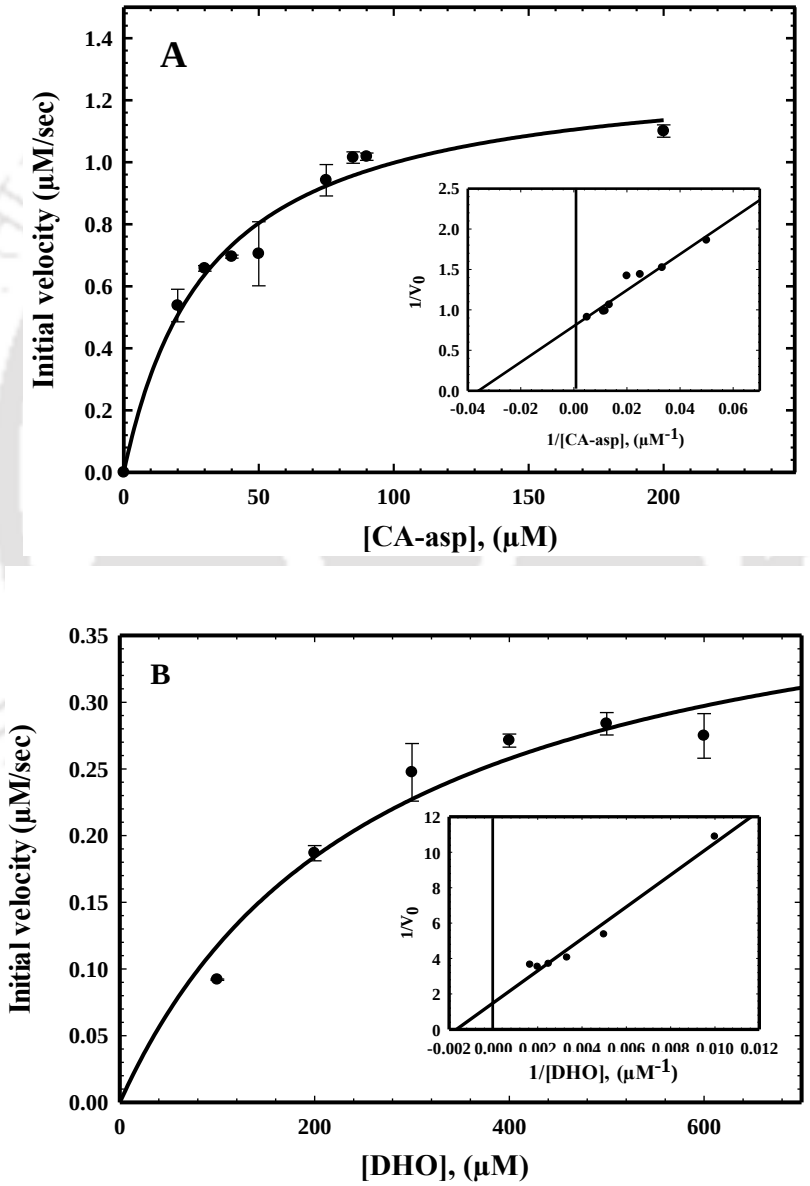


Figure 2.5. Kinetic characterization of *L. donovani* dihydroorotase **(A)** Michaelis-Menten plot for forward reaction **(B)** Michaelis-Menten plot for reverse reaction. Clearly a preference for the forward reaction over reverse reaction has been shown by the kinetics of dihydroorotase enzyme which is also biologically significant. CA-asp: Carbamoyl aspartate, DHO: Dihydroorotate.

DHOase catalyzed reversible reaction is highly dependent on the pH of the buffer used for the forward and reverse reaction, so pH optima was determined for both forward and reverse reactions respectively. The effect of pH on the enzymatic activity was plotted in terms of relative activities at different pH values. A pH optima of 6.0 was observed in the biological direction (forward) while a pH optima of 8.0 was observed in the degradative (reverse) reaction **(Figure 2.6)**.

Table 2.1. Kinetic parameters of *Leishmania donovani* dihydrorootase

Substrate	K_m (μM)	V_{max} (μMs^{-1})	k_{cat} (s^{-1})
CA-asp (Forward)	28.1 ± 6.5	1.2 ± 0.06	2.1 ± 0.10
DHO (Reverse)	220.75 ± 15.3	0.404 ± 0.012	0.67 ± 0.0212

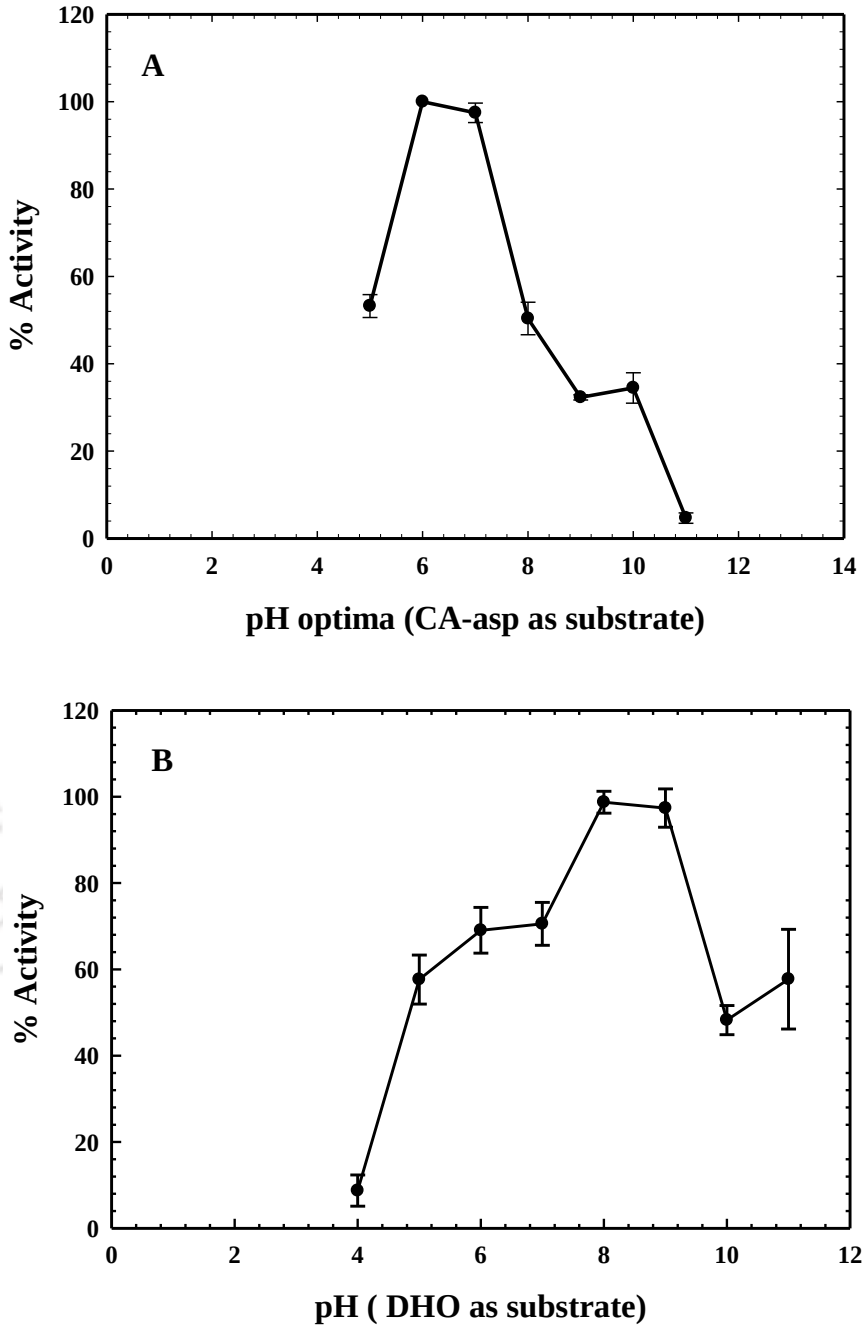


Figure 2.6. pH optima studies on *Ld* dihydroorotase. **(A)** pH optima (6.0) for forward reaction with carbamoyl aspartate as a substrate. **(B)** pH optima (8.0) for reverse reaction with dihydroorotate as a substrate. pH optima studies are of paramount importance for the enzymes of a parasite like *L. donovani* which has a dual existance in the differing pH of sandfly (alkaline) and mammalian phagolysosome (acidic). Forward reaction catalysed by *Ld*DHOase was favored at a slightly acidic pH while the revrese reaction was favored at a slightly alkaline pH. CA-asp: Carbamoyl aspartate, DHO: Dihydroorotate.

2.4 Discussion

The current chemotherapy available for the treatment of visceral leishmaniasis suffers from major drawbacks because of the toxicity and emerging resistance issues with the drugs being used. A fresh look into the unexplored pathways of this protozoan parasite may provide some relief to the existing treatments. Owing to the importance of pyrimidines as the building blocks of nucleic acids, the enzymes of pyrimidine biosynthetic pathway may be vital to the parasitic growth and survival. Thus these enzymes may prove to be good drug targets.

Pyrimidine pathways of *L. donovani* remained unexplored for a long time until Buddy Ullman and coworkers provided a detailed analysis by creating Δ*cps* (carbamoyl phosphate synthetase) null mutants and inhibiting UPRT (uracil phosphoribosyl transferase) enzyme. The Δ*cps* phenotype rendered parasites in an attenuated state resulting in reduced parasite burdens while substrate inhibition of UPRT by uracil resulted in increased growth sensitivity of *L. donovani* pyrimidine auxotrophs *(Wilson et al., 2012).* Till date no group has reported a detailed work on the dihydroorotase (DHOase) of *L. donovani*. The present study aims at functionally validating the role of *LdDHOase* in parasite survival and its implications after drug induced enzyme inhibition. Genes coding for the enzymatic pool of pyrimidine biosynthetic pathway of *L. donovani* are syntenically arranged on the 16th chromosome of *L. donovani* along with the dihydroorotase gene (NCBI Reference Sequence: XM_003859697.1; Gene DB: LdBPK_160580.1) which codes for an enzyme (405 amino acids) with a molecular weight of 43.9 kDa and a pI of 6.0. The parasitic dihydroorotase is 31% identical (83% query coverage) with that of host enzyme with most of the active site residues being conserved. While it showed 63% identity with *Trypanosoma cruzi* (98% query coverage). To carry out characterization and inhibition studies, *Ld*DHOase was overexpressed as a His tagged protein in *E.coli* BL 21(DE3). It migrated on SDS PAGE with a molecular weight of around 48 kDa which classifies *Ld*DHOase as a Type I dihydroorotase, which are presumably of higher molecular weight as compared to class II dihydroorotases like that of *E.coli* (38 kDa) *(Li and Raushel, 2005).*

The forward and reverse reactions catalyzed by dihydroorotases works at different pH. pH optima for *Ld*DHOase followed a similar trend as that observed for mammalian, *Toxoplasma gondii* and *Staphylococcus aureus* counterparts *(Grande-García et al., 2014; Robles Lopez et al., 2006; Truong et. al, 2013).* In the forward direction maximum activity

was observed at pH 6.0 while in the reverse direction maximum activity was observed at pH 8.0 which is in accordance with the previous reports. Slightly lower pH optima of 6.0 has been observed in biological direction in *Ld*DHOase suggesting that the formation of dihydroorotate is favoured in lower pH, one plausible reason for this may be the milieu in which the amastigote form of this parasite lives; i.e in the the phagolysosome of the macrophage which has an acidic environment. While the protozoan parasite also thrives in the milder alkaline environment in sandfly midgut, it would be interesting to observe the rate of the forward reaction in the sandfly stage of parasite. Most possibly the forward reaction in the sandfly stage must proceed with albeit at a slower rate as compared to milder acidic conditions. The reverse reaction has lesser biological implications as it has lesser physiological relevance as the pyrimidine pathway always proceeds in the direction of synthesis, also the accumulation of substrate always forces the reaction to move in the forward direction, and as soon as the product is formed it is used up in the form of substrate by the next enzyme in the pathway, thus the chances of product accumulation being rare the reaction is never pushed to degradative (reverse) direction. Unless the next enzyme in the pathway is inhibited the feasibility of the reverse reaction remains dim. But the structure of dihydoorotase and same active site orientation for both substrates prompted us to characterize it in both directions.

The ring cyclization reaction catalyzed by dihydroorotase proceeded with a K_m of 28.16 μM, while the ring cleavage or N-carbamoyl aspartate formation had a K_m of 220.75 μM. The lower K_m value for forward reaction clearly indicates that formation of dihydroorotate is favoured upon N-carbamoyl aspartate which is also biologically observed, as the enzyme has high affinity towards N-carbamoyl aspartate. This can be physiologically implied in the context of *L. donovani* as it resides in the phagolysosome of macrophage in acidic environment which has a pH closer to that observed in the pH optima studies (6.0) thus favoring the dihydroorotate formation and requiring lesser carbamoyl aspartate. There may be a possibility that the acidic environment of phagolysosome has contributed in favoring the biosynthesis of dihydroorotate thus signifying lesser K_m value. At acidic pH the reaction is favored in biological direction only, the condition which is not present usually in case of mammals which maintain a physiological pH inside the cell thus requiring more of N-carbamoyl aspartate (K_m = 241.2 μM) *(Grande-García et al., 2014)* to operate the reaction,

this is in strike contrast with *L. donovani* in which acidic environment (in the phagolysosome) required for the formation of dihydroorotate is naturally present thus requiring lesser N-carbamoyl aspartate (K_m = 28.16 μM). Similarly for the reverse reaction *Ld*DHOase requires more of dihydroorotate (K_m = 220.75 μM) as compared to Human counterpart (K_m = 27.8 μM) as the pH optima for *Ld*DHOase is at pH 8.0.

Existance of monomeric form of dihydroorotase has been reported previously in most of the parasites including *P.falciparum, P.berghei* and *T.gondii (Krungkrai et al., 2008; Krungkrai et al., 1990; Robles Lopez et al., 2006)* while dimeric forms exist in the bacteria like *E.coli (Lee et al., 2005)* and *B.anthracis (Mehboob et al., 2010).* In humans too the monomeric form is present as a part of a multifunctional enzyme. However a tetrameric structure for *Ld*DHOase was revealed by size exclusion chromatography. It is clearly evident from the size exclusion data that most of the protein lies in the dimer fraction and lesser in the tetrameric fraction. It can be presumed that probably the dimer and tetramer maintain equilibrium in the solution. If so, both the dimeric and tetrameric forms of the enzyme must be active as the active site lies in the core of the protein structure and not on the dimer interface.

2.5 Conclusion

Dihydroorotase, the third enzyme of the *de novo* pyrimidine biosynthetic pathway is structurally distinct from its mammalian counterpart. The dihydroorotase mediated synthesis (forward) reaction was favored over degradative (reverse) reaction as evident from the kinetic data. Also the forward reaction exhibited maximum activity at a mild acidic pH while in contrast the reverse reaction at a slightly alkaline pH exhibited maximum activity. Dihydroorotase exhibited tetrameric structure while a large fraction lies in the dimeric state. Dihydroorotase is a promising enzyme for drug targeting and can be useful for studying the pyrimidine pathway in general.

CHAPTER 3

Virtual screening of *L. donovani* dihydroorotase inhibitors using *in silico* approaches*

ABSTRACT

Dihydroorotase, a crucial enzyme of the *de novo* pyrimidine biosynthesis pathway of *L. donovani* was investigated using *in silico* approaches which provided critical insights on the structural aspects and also revealed potential lead compounds which could be used as inhibitors. Dihydroorotase substrates: carbamoyl aspartate and dihydroorotate were bound to the active site of the enzyme with binding energies of -2.72 kJ/moles and -4.1 kJ/moles respectively. While docking studies with *L. donovani* dihydroorotase also revealed biotin sulfone and kaempferol as the potential lead compounds which were bound to the loop forming residues of the *L. donovani* dihydroorotase with binding energies of -6.4 kJ/moles and -4.56 kJ/moles respectively. These *in silico* findings may help in developing therapeutic interventions for the treatment of visceral leishmaniasis.

*Part of the work has been published in Biochemie, 2016, 131, 45-53.

3.1 Introduction

Development of effective drugs against visceral leishmaniasis has been a challenging task. Coming up with a specific drug which targets the parasite and spares mammalian cells is difficult to achieve. A comparison of mammalian and parasitic metabolic pathways provides key insights which aid in the drug discovery process. In the current study dihydroorotase enzyme (*de novo* pyrimidine biosynthesis pathway) owing to structural dissimilarities with mammalian counterpart has been targeted for drug efficacy.

Several compounds have been reported as inhibitors of bacterial DHOase that are used as antimicrobial agents *(Li and Raushel, 2005)*. In the past years substrate analogues and transition state analogues of DHOase like HDDP (2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate) and TDHO (6-L-thiodihydroorotate) have been synthesized and successfully used as mammalian DHOase inhibitors *(Brooke et al., 1990)*. Earlier reports have used carbamoyl aspartate analogues and boronic acid transition state analogues as inhibitors on murine leukemia cell lines *(Kinder et al., 1990)*. Li and Raushel have synthesized 4, 6-dioxo-piperidine-2(S)-carboxylic acid as an *E.coli* DHOase inhibitor *(Li and Raushel, 2005)*. Myricetin and kaempferol which are flavonols showed significant inhibition of *Klebsiella pneumoniae* DHOase *(Peng and Huang, 2014)*. N-phosphonacetyl-L-aspartate (PALA) inhibits the activity of aspartate transcarbamylase (ATCase) at the second step of *de novo* pyrimidine biosynthetic pathway and has been successfully used as the inhibitor of this pathway. Several other inhibitors including Brequinar, Leflunomide and Atovaquone are used as potential inhibitors of *de novo* pyrimidine biosynthetic pathway *(Christopherson et al., 2006)*.

There are significant structural differences at active site as well as low sequence similarity between DHOase from human and *L. donovani.* While, the human DHOase is a part of a multifunctional enzyme complex known as CAD (Carbamoyl-phosphate synthetase II, Aspartate transcarbamoylase and Dihydroorotase) which catalyzes the first three steps of the *de novo* pyrimidine biosynthetic pathway *(Coleman et al., 1977: Porter et al., 2004: Evans and Guy, 2004)*. The DHOase from *L. donovani* is a single enzyme catalyzing the third step of the pathway. Further, our initial analysis indicates significant structural differences at active site as well as low sequence similarity. The existing structural difference between the host and parasitic dihydroorotase (DHOase) makes this enzyme as a potential drug target.

3.2 Materials and methods

3.2.1 Analysis of L. donovani dihydroorotase

The nucleotide and amino acid sequences of *L. donovani* dihydroorotase were retrieved from Gene DB and NCBI databases. Multiple sequence alignments were done by NCBI BLAST, Clustal Omega and ESPript 3.0 *(Robert and Gouet, 2014)*. A schematic representing the workflow for the *in silico* studies done on *L. donovani* dihydroorotase are given in **Figure 3.1**.

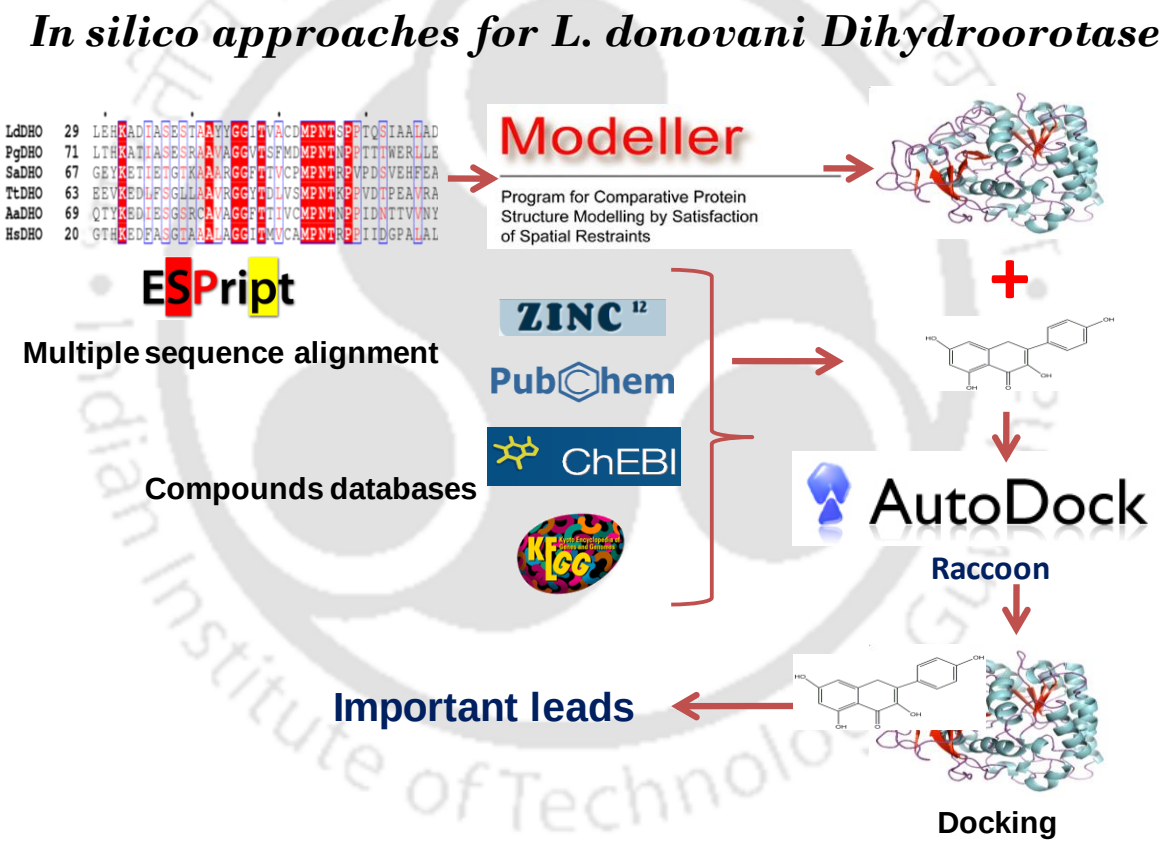


Figure 3.1. Schematic representing the workflow for the *in silico* studies done on *L. donovani* dihydroorotase. The structurally distinct dihydroorotase enzyme of the *de novo* pyrimidine biosynthesis pathway was modelled using homology based modelling employing Modeller 9.12. The modelled *Ld* DHOase was further screened for potential lead compounds using Autodock program. The identified lead compounds were further tested for their inhibitory effects on the recombinant *Ld* DHOase.

3.2.2 Homology modeling of L. donovani dihydroorotase

Amino acid sequence of *Ld*DHOase protein, accessed from Gene DB (LdBPK_160580.1) was aligned using BLASTp multiple sequence alignment program to obtain highly similar sequences *(Altschul et al., 1997)*. Structural coordinate files for highly similar structures [PDB IDs: 2GWN (*Porphyromonas gingivalis*), 3GRI (*Staphylococcus aureus*), 2Z00 (*Thermus thermophilus*), 3D6N (*Aquifex aeolicus*)] were downloaded from PDB (www.rcsb.org) and were used as templates for model predictions. Multiple sequence alignment of *Ld*DHOase with the template sequences as well as with human DHOase was done by ESPript 3.0 *(Robert and Gouet, 2014)*. Modeller 9.12 was used for the generation of predicted model structure of *Ld*DHOase using template structures *(Webb and Sali, 2014)*. The model was stereochemically verified using Procheck *(Laskowski et al., 1996)* by generating ramachandran map and also by visualizing it in pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrodinger, LLC). Energy minimization was done using Swiss PDBViewer *(Guex and Peitsch, 1997)*. The modeled structure of the protein has been submitted to protein model database available on https://bioinformatics.cineca.it/PMDB/ (submission ID: PM0080560).

3.2.3 Docking studies on L. donovani dihydroorotase

The energy minimized structure of *Ld*DHOase was used in Autodock program for virtual screening of inhibitors *(Morris et al., 2009)*. Structural analogue based docking approach was followed in which structures having close resemblance with natural substrates were chosen. Autodock version 1.5.4 and Raccoon were used to run the docking program (center grid box: x center: 18.314, y center: 17.615, z center: 15.557, Lamarckian genetic algorithm and number of runs: 100). To validate the authenticity of the docking process, firstly the natural substrates (carbamoyl aspartate and dihydroorotate) of dihydroorotase were docked to the modeled enzyme. Human DHOase was also docked with same inhibitors to compare the change in binding energies. Binding energies (ΔG) were used as a measure to select potential compounds. Three dimensional structures of compounds (mol format) for screening were retrieved from Pubchem, KEGG compounds and Zinc database respectively *(Kim et al., 2016: Irwin and Shoichet, 2005)*.

3.3 Results

3.3.1 Analysis of L. donovani dihydroorotase

The coding sequence for *L. donovani* dihydroorotase (Gene DB: LdBPK_160580.1) is present on chromosome no. 16 (diploid). The 1218 bp coding sequence encodes a 405 amino acid protein with a predicted pI of 6.0 and mass of 43.9 kDa. The dihydroorotase enzyme comprises of an amidohydrolase domain which spans most of the protein length. Thus dihydroorotase belongs to the metallo dependent hydrolases superfamily. All the genes coding for the enzymes of pyrimidine pathway are located on the same locus on chromosome no. 16 which is referred to as synteny. In order to validate dihydroorotase as a potential drug target, it was compared with Host counterpart. A BLAST (Basic Local Alignment Search Tool) analysis of *Ld*DHOase and *Hs*DHOase revealed 34% identity (97% query cover) **(Figure 3.2)**. A comparison of active site residues tells us that the catalytic residues are conserved but many have been replaced. *Homo sapiens* DHOase active site is lined with basic residues like His1473, Arg1661, His 1690 and a single Asp 1686 acidic residue, and also neutral residues like Asn1505 and Pro 1702 while *Ld*DHOase active site is also lined with neutral residues like Thr 125, Gly 126, Ala 282, Asn 253, Ala 296. Few basic residues like His23, Arg 25, His 284 and Asp52 as a single acidic residue are also present **(Figure 3.3)**. Asp52 in the *Ld*DHOase has been replaced by Ala1592 in the *Hs*DHOase. But the same covalent bonding with carbamoyl aspartate is contributed by Asp 1686 of *Hs*DHOase as is done by Asp 52 of *Ld*DHOse. *Hs*DHOase active site is more of basic while *Ld*DHOase active site is a mix of basic, acidic and neutral amino acid residues. Also the residues involved in the loop formation are also different like in *Ld*DHOase Thr 125, Gly 126 form the loop region while Phe 1561 and Ser 1562 form the loop in *Hs*DHOase. The loop residues are crucial in maintaining the closed and open loop states of the enzyme and thus affect the catalysis. Thus it is clearly evident from the active site analysis that the active sites of both host and parasitic counterparts are significantly different and thus the parasitic enzyme can be drug targeted.

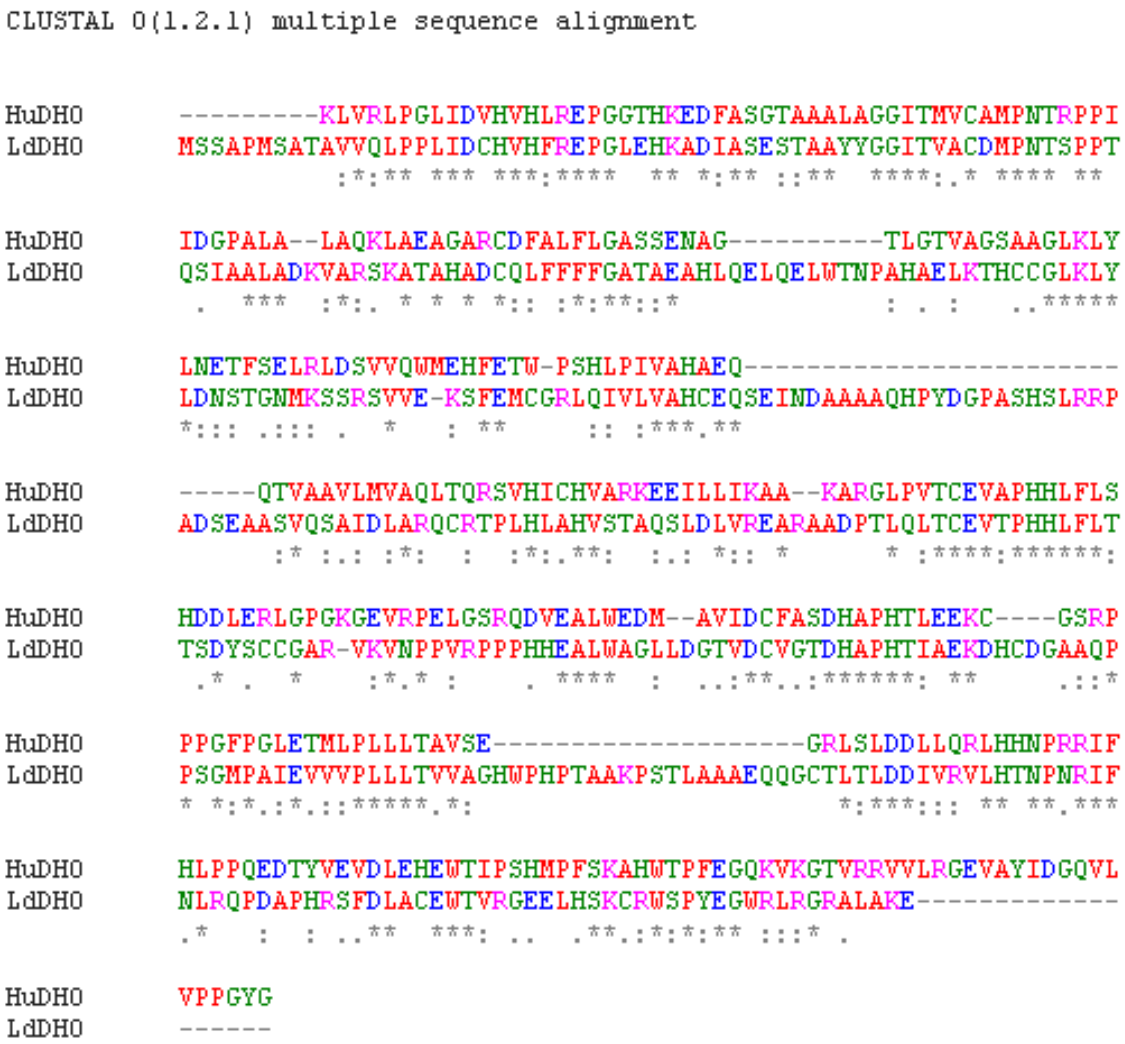


Figure 3.2: Multiple sequence alignment of *Homo sapiens* and *Leishmania donovani* dihydroorotase. Boxed regions represent conserved regions while the arrow represents differences in the residues. Comparison of amino acid sequence of dihydroorotase from *Leishmania donovani* parasite and *Homo sapiens* revealed crucial differences in the active site residues as well in the loop region which directs the activity of enzyme.

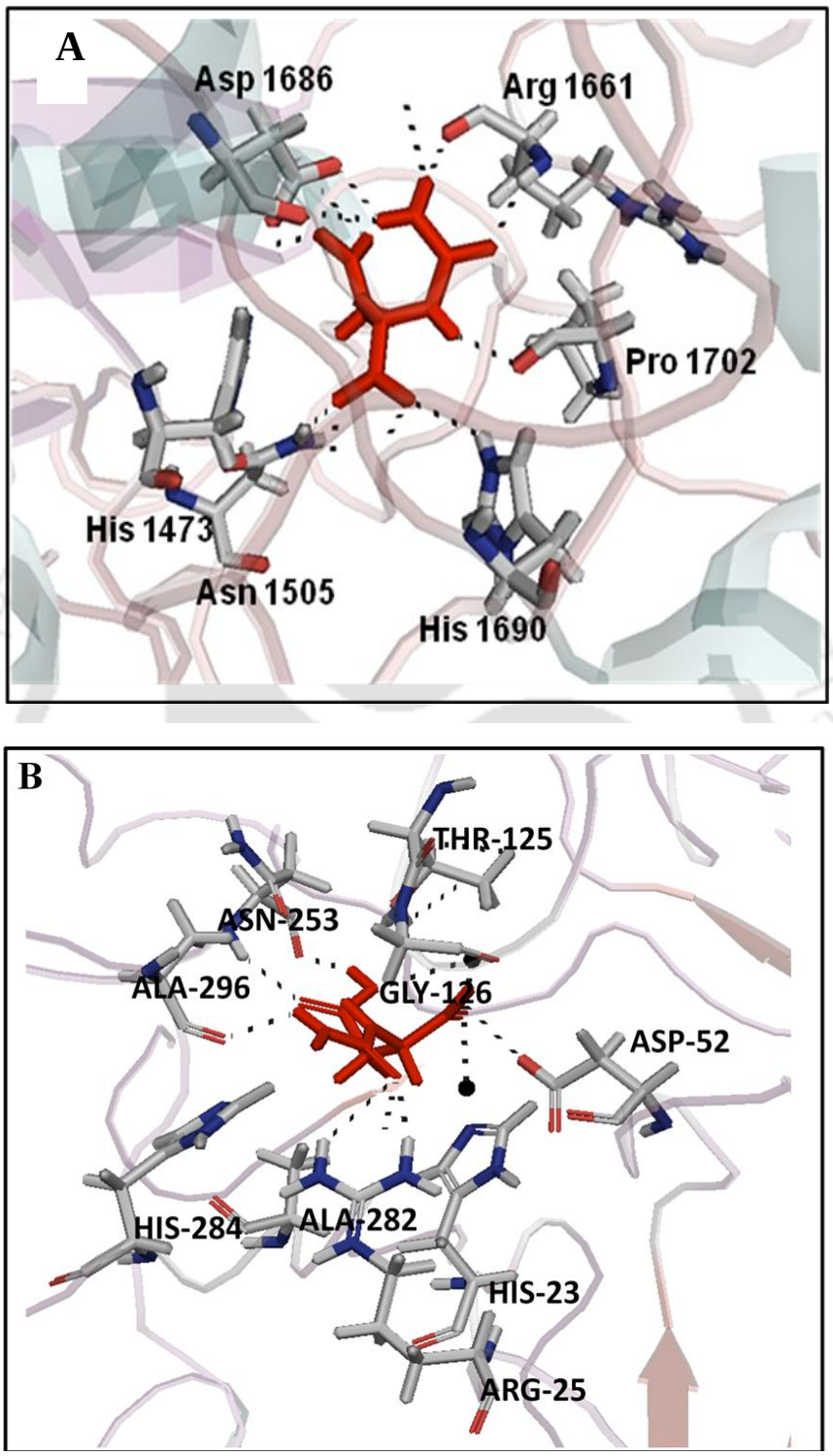


Figure 3.3: Active site differences between **(A)** *Homo sapiens* and **(B)** *Leishmania donovani* dihydroorotase. A comparison of amino acid residues lining the active site of *Homo sapiens* and *Leishmania donovani* dihydroorotase using the crystal structure of Human DHOase (4C6J) and modelled structure of *Ld* DHOase. The active sites of both the counterparts have significant differences.

3.3.2 Homology modeling of L. donovani dihydroorotase

Multiple sequence alignment of *Ld*DHOase with the template sequences and with human DHOase revealed conserved regions **(Figure 3.4)**. The modeled structure of *Ld*DHOase superimposed with *Hs*DHOase is depicted in **Figure 3.5**. According to ramachandran plot statistics 85.8% residues were placed in the most favored regions, 11.3 % were in additional allowed regions, 2.3 % in generously allowed regions and 0.6% were in disallowed regions **(Figure 3.6)**. Thus the model was of good quality and could be used in docking and simulations. The modeled structure of the protein has been submitted to protein model database available on https://bioinformatics.cineca.it/PMDB/ (submission ID: PM0080560). The conserved active site residues Arg25, Asn55, Thr125, Gly126, Asn253, Ala282, His284, and Gly294 of the modelled enzyme are depicted. Zinc atoms were surrounded by active site residues His21, His 23, Asp52, Lys111 and His281 respectively.

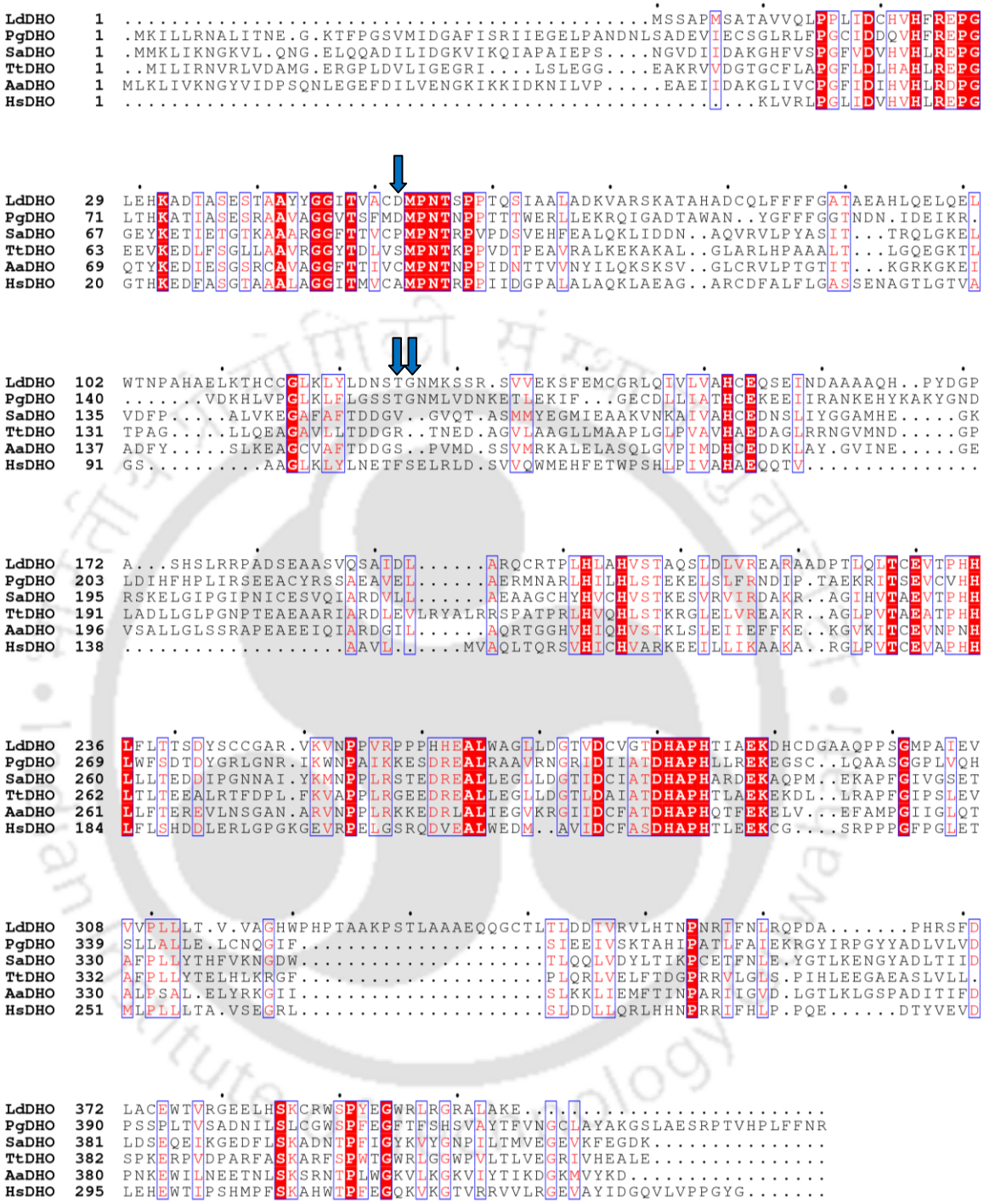


Figure 3.4. Multiple sequence alignment of *L.donovani* dihydroorotase with *P. gingivalis* (2GWN), *T. thermophilus* (2Z00), *S. aureus* (3GRI), *A.aeolicus* (3D6N) and *Homo sapiens* DHOase (4C6C). Pointed arrowheads are representing significant differences in active site residues of *L. donovani* and *Homo sapiens* DHOase.

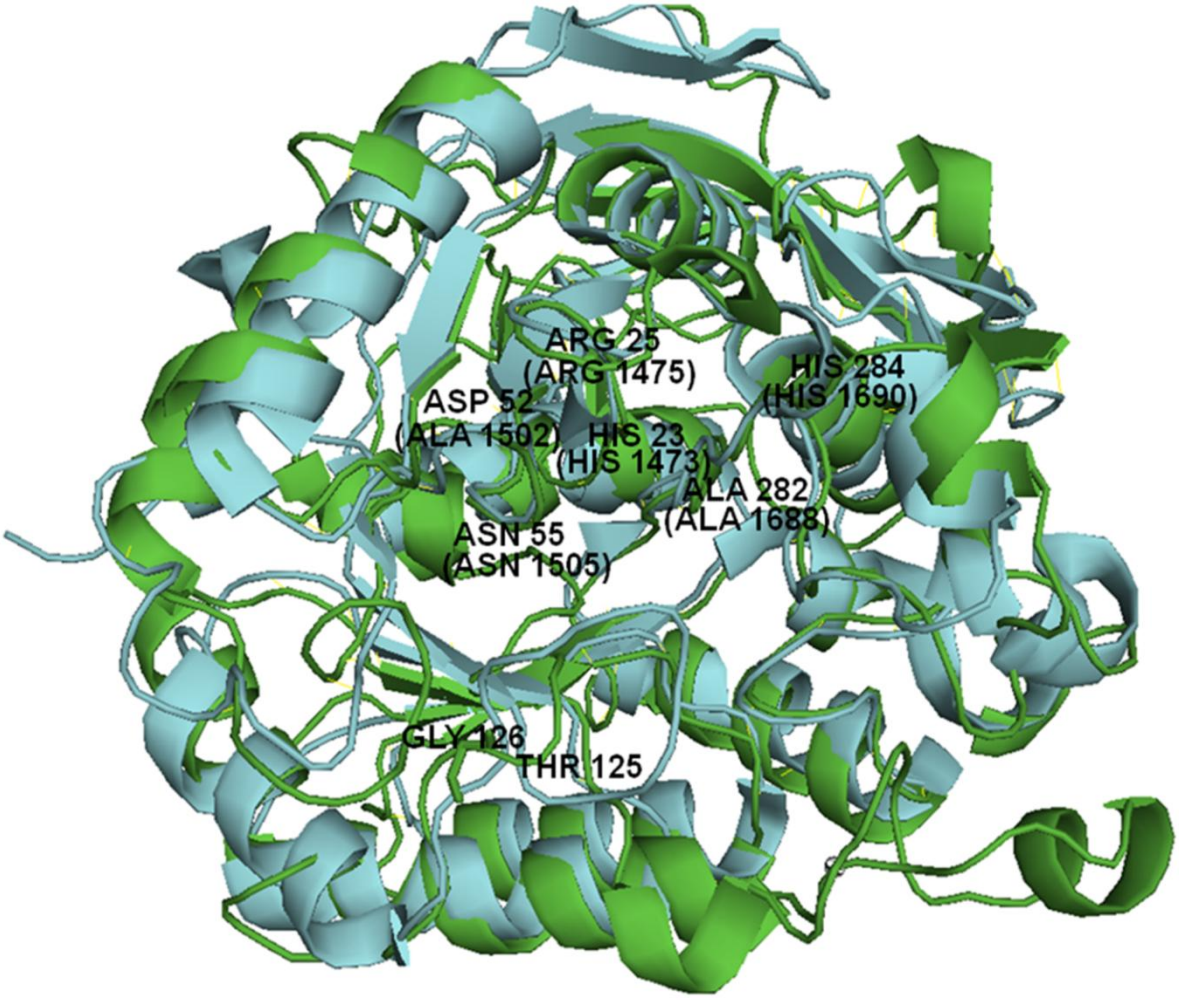
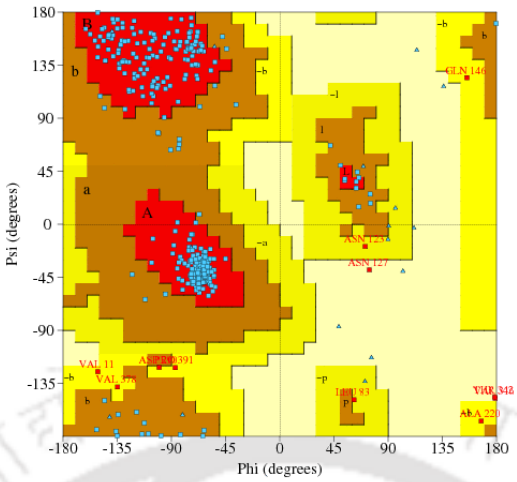


Figure 3.5. *Leishmania donovani* dihydroorotase modeled structure (Green) superimposed on *Homo sapiens* dihydroorotase (4C6C) (Cyan) displaying the active site residues. *Hs*DHOase residues are depicted in bracket. Asp52 in *Ld*DHOase has been replaced by Ala1502 in *Hs*DHOase, also significant difference in loop residues (Thr125 and Gly126) are evident.



Ramachandran Plot statistics	No. of residues	%-tage
Most favoured regions	303	85.8%
Additional allowed regions	40	11.3%
Generously allowed regions	8	2.3%
Disallowed regions	2	0.6%

Figure 3.6: Ramachandran plot for Modelled *Ld* dihydroorotase depicting the allowed and disallowed regions. The quality of the modelled structure of *Ld* dihydroorotase was verified by ramachandran plot. Only 0.6 % residues were depicted in disallowed region while 85.8% of the residues were in the most favored regions. Ramachandran plot statistics depicts the modelled *Ld* dihydroorotase of good stereochemical quality which could be further employed for various *in silico* studies.

3.3.3 Docking studies on L. donovani dihydroorotase

Carbamoyl aspartate was bound to the active site of dihydrorootase with a binding energy of-2.72 kJ/mol while dihydroorotate was bound to the active site with a binding energy of -4.12 kJ/mol **(Figure 3.7)**. Both the substrates were bound to the active site through His 23, Arg 25, Asp52, Thr 125, Gly 126, Asn 253, Ala 282, His 284 and Ala 296 residues. Binding energies (ΔG) were used as a measure to select potential compounds. Three dimensional structures of compounds (mol format) for screening were retrieved from Pubchem, KEGG compounds and Zinc database respectively *(Kim et al., 2016: Irwin and Shoichet, 2005)* **(Table 3.1)**. Various compounds were screened and their binding energies compared with natural substrates. Top ranked compounds were selected based on their binding energies

(Table 3.7). The top ranked compounds included biotin sulfone (-6.4 kJ/moles), biotin sulfoxide (-5.87 kJ/moles), lydimycin (-5.46), 6-[5-[(3aS,4R,6aS)-2-oxo-1,3,3a,4,6,6a-hexahydrothieno[3,4-d]imidazol-4-yl] pentanoylamino] hexanoate (-5.38) and biotin-epsilon-aminocaproic acid (-5.27 kJ/moles) **(Table 3.1)**. Among the docked compounds biotin sulfone displayed a binding energy of -6.4 kJ/moles while kaempferol showed a binding energy of -4.9 kJ/moles with *Ld*DHOase. Kaempferol did not show any binding with human DHOase while biotin sulfone showed binding with human DHOase as well (-4.56 kJ/moles). Interestingly, in a previous study, kaempferol has also been found to inhibit *Klebsiella pneumoniae* DHOase in an uncompetitive manner *(Peng and Huang, 2014)*. Although, there were few better options of inhibitors indented *in silico* **(Table 3.1)**, these compounds (kaempferol and biotin sulfone) were chosen due to commercial availability.

Notably kaempferol and biotin sulfone displayed binding away from the active site but with key loop residues Thr 125, Gly 126. In addition to loop residues biotin sulfone also contacted Tyr 120, Leu121, Asp 122, Ser 124, Lys 129, Ser 130, and Ser 150. Kaempferol contacted Asp 122, Asn 123, Ser 124, Thr 125, Gly 126, Lys 129, Ser 130 and Ser 156. Binding of kaempferol and biotin sulfone to the loop residues of the dihydroorotase may critically impair the catalytic activity of the enzyme because as discussed in the chapter 1 and also in previous sections the loop movement directs the loop in and loop out confirmation which further directs the enzymatic activity. Any change in the spatial orientation of the loop region is supposed to hamper enzymatic activity which has also been supported from enzyme kinetics data **(Figure 4.1)**. Thus owing to their binding to key residues of the loop regions of the dihydroorotase along with their binding affinities kaempferol and biotin sulfone were selected for further studies on recombinant *Ld*DHOase.

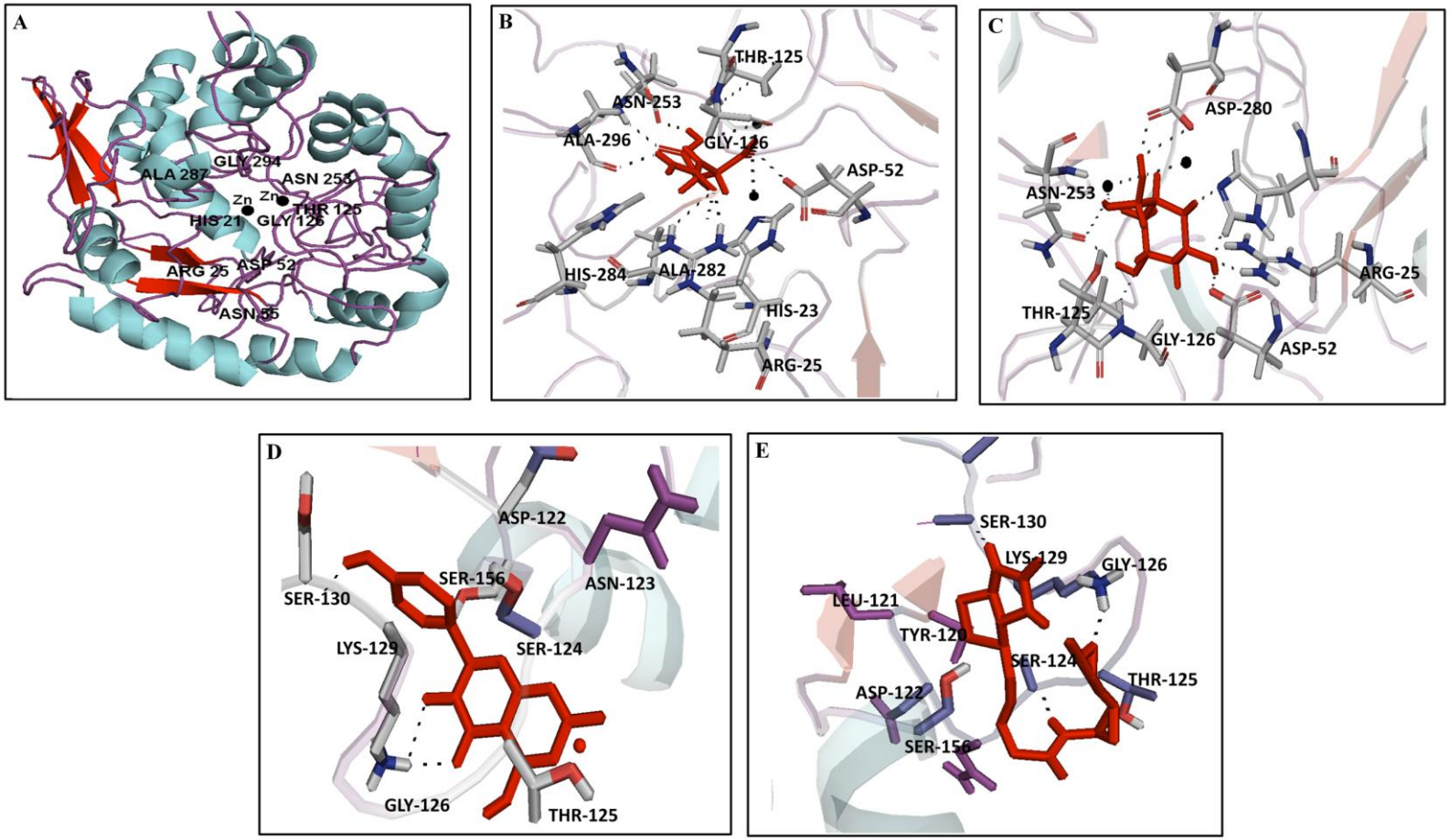
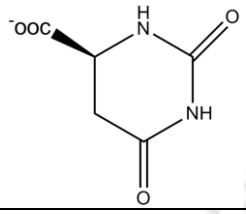
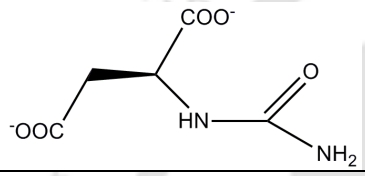
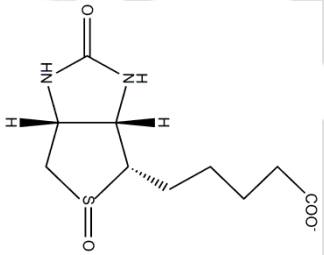
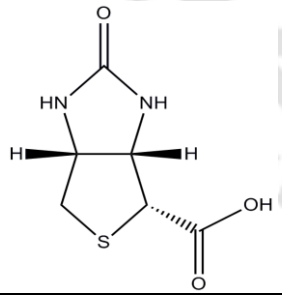
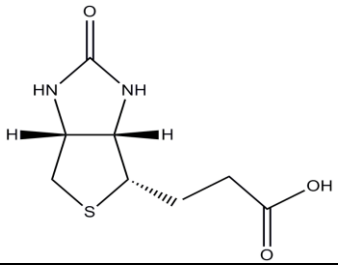
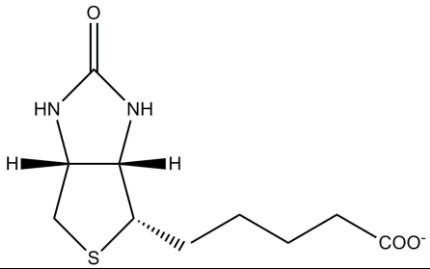
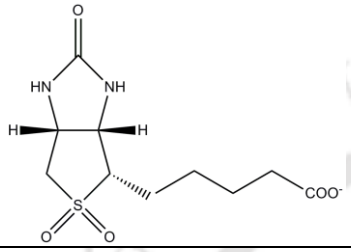
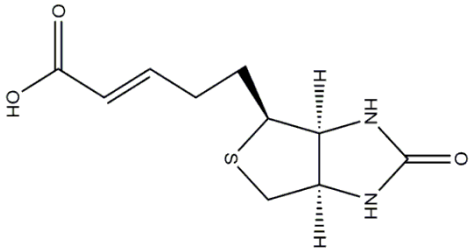
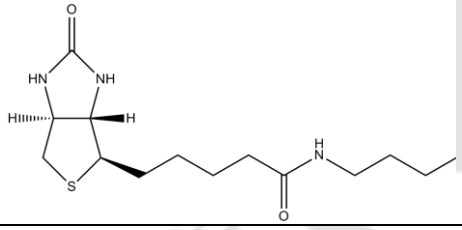
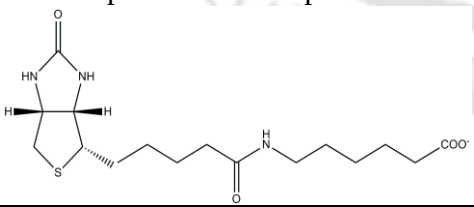
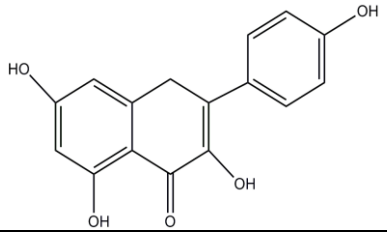


Figure 3.7. *Insilico* studies on *L. donovani* dihydroorotase **(A)** Homology modeling of *Ld*DHOase showing the active site residues. **(B)** Carbamoyl aspartate (substrate) docked in the active site of *Ld*DHOase, Binding energy: -2.72 kJ/mol. **(C)** Dihydroorotate (substrate) docked in the active site of *Ld*DHOase, Binding energy: -4.1 kJ/mol **(D)** Kaempferol (inhibitor) docked to *Ld*DHOase, Binding energy: -4.9 kJ/mol **(E)** Biotin sulfone (inhibitor) docked to *Ld*DHOase, Binding energy: -6.4 kJ/mol

Table 3.1: Structure and binding energies of compounds docked with modeled *Ld*DHO

S.No.	Compound	Lowest binding energy with *Ld*DHO kJ/moles	Lowest binding energy with Human DHO kJ/moles
1	Dihydroorotate 	-4.1	-3.65
2	Carbamoyl aspartate 	-2.72	-3.66
3	Biotin sulfoxide 	-5.87	-4.37
4	Tetranorbiotin 	-5.01	-3.22
5	Bisnorbiotin 	-4.94	-3.9

6	Biotin 	-5.22	-4.19
7	Biotin sulfone 	-6.4	-4.56
8	Lydimycin 	-5.46	-4.15
9	6-[5-[(3aS,4R,6aS)-2-oxo-1,3,3a,4,6,6a-hexahydrothieno[3,4-d]imidazol-4-yl]pentanoylamino]hexanoate(25322995) 	-5.38	-0.93
10	Biotin-epsilon-aminocaproic acid 	-5.27	No binding
11	Kaempferol 	-4.9	No binding

3.4 Discussion

Transition state analogues and substrate analogues of dihydroorotase have been tested as potential inhibitors of dihydroorotase in the past. *(Brooke et al., 1990 and Kinder et al., 1990)*. In the present study virtual screening of inhibitors revealed biotin derivatives and kaempferol to be the potential inhibitors of *Ld*DHOase. Being structural analogues they were intended to bind the active site but instead they were bound to critical loop forming residues of *L. donovani* dihydroorotase. The loop forming residues of dihydroorotase acts as a switch in maintaining active and inactive states of the enzyme. As the movement and orientation brings the enzyme to loop in and loop out confirmations, thus it becomes interesting to see what consequences are observed upon binding of biotin sulfone and kaempferol to the loop residues.

3.5 Conclusion

In silico studies on *L.donovani* dihydroorotase revealed the presence of a characteristic beta barrel fold, hallmark of amido hydrolases along with conserved active site residues. Also key differences in the active site residues were evident between the mammalian and parasitic enzyme counterparts. Numerous compounds were docked to *Ld* dihydroorotase of which kaempferol and biotin sulfone emerged out as strong hits and were considered to be potential inhibitors of *L. donovani* dihydroorotase based on their binding energies.

CHAPTER 4

Physiological implications of inhibitors on *de novo* and salvage pyrimidine pathway of *L. donovani**

ABSTRACT

In the present work the inhibitory effects of two compounds (kaempferol and biotin sulfone) identified through *in silico* approaches was verified on recombinant *Ld*DHOase. Kaempferol and biotin sulfone inhibited *Ld*DHOase in a mixed (K_i: 151.4 μM) and uncompetitive manner (K_i: 55.4μM). Further these compounds were tested for their antileishmanial potential. Supposedly a parallel salvage pathway prevented the substantial cell death which was again verified by qRT PCR analysis which revealed that both pathways reciprocated each other when one was inhibited. Blockages of either *de novo* or salvage pathway alone or in combination by respective inhibitors also lead to substantial DNA damage. The data presented in the current work has implications for the pathway inhibition and analyses.

*Part of the work has been published in Biochemie, 2016, 131, 45-53.

4.1. Introduction

Developing an effective treatment regimen for visceral leishmaniaisis is still a daunting task for investigators. Inhibition of pathways still remains a feasible choice by which better treatment options can be developed for visceral leishmaniaisis. Various metabolic pathways especially the redox pathway of the parasite has been targeted and effective leads has been deciphered which promises to bring the visceral leishmaniasis cases to negligible numbers. Still various other pathways are being investigated. The pyrimidine and purine pathways garner specific interest in the research community as the parasite lacks *de novo* pathway for purines but has both *de novo* and salvage routes for pyrimidine synthesis. However growing body of literature suggests that pyrimidine pathway can be selectively targeted to develop effective drugs. Going by the same idea in the current study effect of two inhibitors was studied on purified dihydroorotase, and pyrimidine pathways of the protozoan parasite *L. donovani*.

Pyrimidine biosynthesis pathway is well developed in *L. donovani (Wilson et al., 2012)*. Pyrimidines in *L. donovani* can be formed *via* both *de novo* and salvage pathways *i.e.* parasite is prototrophic to pyrimidines while it is auxotrophic to purines. The *de novo* pyrimidine biosynthetic pathway has been well deciphered in *L. donovani (Wilson et al., 2012).* Thus, the inhibition of the *de novo* pyrimidine biosynthetic pathway of *L. donovani* by inhibiting DHOase may cause loss of infectivity. Interestingly, we did not find much effect of DHOase inhibition on survival of the pathogen suggesting that the salvage pathway may supplement the pyrimidine biosynthesis. Studies on inhibition of *de novo* or salvage pathways alone and both pathways together have improved our understanding of the pyrimidine biosynthesis pathway in *L. donovani.* In the present study the *de novo* and salvage pathways of the *L. donovani* parasite were investigated using their respective inhibitors and the contribution of both pathways in the growth and survival of parasite was analyzed.

4.2 Materials and methods

4.2.1 Materials

Leishmania donovani strain BHU 1081 was a kind donation from Prof. Shyam Sundar, Banaras Hindu University, India. The culture conditions for *L. donovani* are well established in our laboratory *(Saudagar et al., 2013, Das et al., 2015).* In brief, the *L. donovani* was grown in

M199 medium supplemented with 15% heat inactivated fetal bovine serum (FBS), 100Uml^{-1} and 100 μgml^{-1} streptomycin. For inhibition and gene expression based experiments M199 media was supplemented with 15% dialyzed heat inactivated fetal bovine serum. The DreamTaq Green DNA polymerase was purchased from Thermo scientific. All restriction enzymes were purchased from ILS (NEB). Isopropyl β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Merck. *N*-carbamoyl-L-aspartic acid (CA-asp) was ordered from MP Biomedicals and L-dihydroorotate (DHOase) and kaempferol were ordered from Sigma Aldrich Chemicals. Biotin sulfone was purchased from Enamine. All other chemicals were of highest purity grade, procured from Sigma Aldrich or Merck.

4.2.2 Inhibition studies on L. donovani dihydroorotase

Kaempferol and biotin sulfone which showed promising binding affinity to the modelled enzyme were used for enzyme inhibition studies. Briefly 100 μM of kaempferol or biotin sulfone were pre-incubated with 0.6 μM enzyme for 15 minutes. Varying concentrations of dihydroorotate (80μM to 500μM) were then added to the reaction mixture to initiate the reaction respectively for 10 minutes. Absorbance values were recorded at 230 nm. All reactions were performed in triplicates and the reactions were carried out at 25°C. Kinetic parameters (K_m and V_{max}) and inhibition constant (K_i) were estimated from the double-reciprocal plots.

4.2.3 Anti-leishmanial effect of the inhibitors of de novo and salvage pathways enzyme(s)

Parasite survival (cell viability) upon treatment with kaempferol and biotin sulfone was estimated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay *(Mossman, 1983)*. Briefly exponential phase *L. donovani* promastigotes (2.5×10^6) cultivated in M199 media supplemented with 15% dialyzed heat-inactivated fetal bovine serum (FBS), were seeded in 96 well plate with varying concentrations of kaempferol and biotin sulfone (50 μM to 500 μM) incubated at 25°C for 24 hrs. Cells were pelleted down at 1500 xg, re-suspended in MTT and incubated further for 4 hrs. Cells were finally pelleted down and formazan pellet re-suspended in DMSO and absorbance measured at 570 nm. Miltefosine (IC_{50} of 25μM) was used as a positive control throughout the experiments *(Paris et al., 2004)*. The bar graph was plotted using Sigma plot (Systat Software, San Jose, CA).

4.2.4 Quantitative Real time PCR

Total RNA was isolated from treated and control *L. donovani* using RNeasy minikit (Qiagen). Equal amount of total RNA (quantified using Biospectrometer) was used for first strand cDNA synthesis using AMV First Strand cDNA synthesis kit (NEB) according to manufacturer's protocol. qRT-PCR was carried out using Power SYBR® Green PCR Master mix (Invitrogen) in a thermal cycler 7500 Real Time PCR System (Applied Biosystems) in triplicates. The data generated was normalized to alpha tubulin (endogenous control) and fold change was calculated using $2^{-\Delta\Delta Ct}$ method. All the primers used in the experiment are listed in **Table 4.1** *(Livak and Schmittgen, 2001).*

4.2.5 DNA content analysis

Flow cytometer based analysis was performed in which a DNA binding fluorophore propidium iodide (PI) was employed to analyze the cell populations *(Bertuzzi et al., 1990)*. Briefly, log phase promastigotes were treated with 500 µM of kaempferol and zebularine (a salvage pathway inhibitor) *(Lemaire et al., 2009)* alone and in combination for 24 hrs, while promastigotes grown in 15% FBS and 15% dialyzed FBS served as controls. Following 24 hrs, cells were washed twice with PBS and finally re-suspended in PBS, fixed in 70% methanol and kept overnight at -20°C. The following day cells were again PBS washed twice to remove methanol and finally re-suspended in PBS followed by RNAse and PI treatment in dark at 37°C. PI treated cells were subjected to flow cytometer (BD FACS Calibur) after 30 minutes of incubation. The area under the peaks was calculated by ModFitLT^{TM} software package.

4.3 Results

4.3.1 Inhibition studies on L. donovani dihydroorotase

A simple hyperbolic kinetics was observed when either of the substrate was varied. The data was re-plotted in the form of Lineweaver-Burk plot (LB-Plot) and analyzed. The pattern of LB-Plot indicates mixed enzyme inhibition by kaempferol on binding to *Ld*DHOase with a K_i of 151.4 µM while biotin sulfone displayed uncompetitive inhibition with a K_i of 55.4 µM **(Figure 4.1A and 4.1B)**.

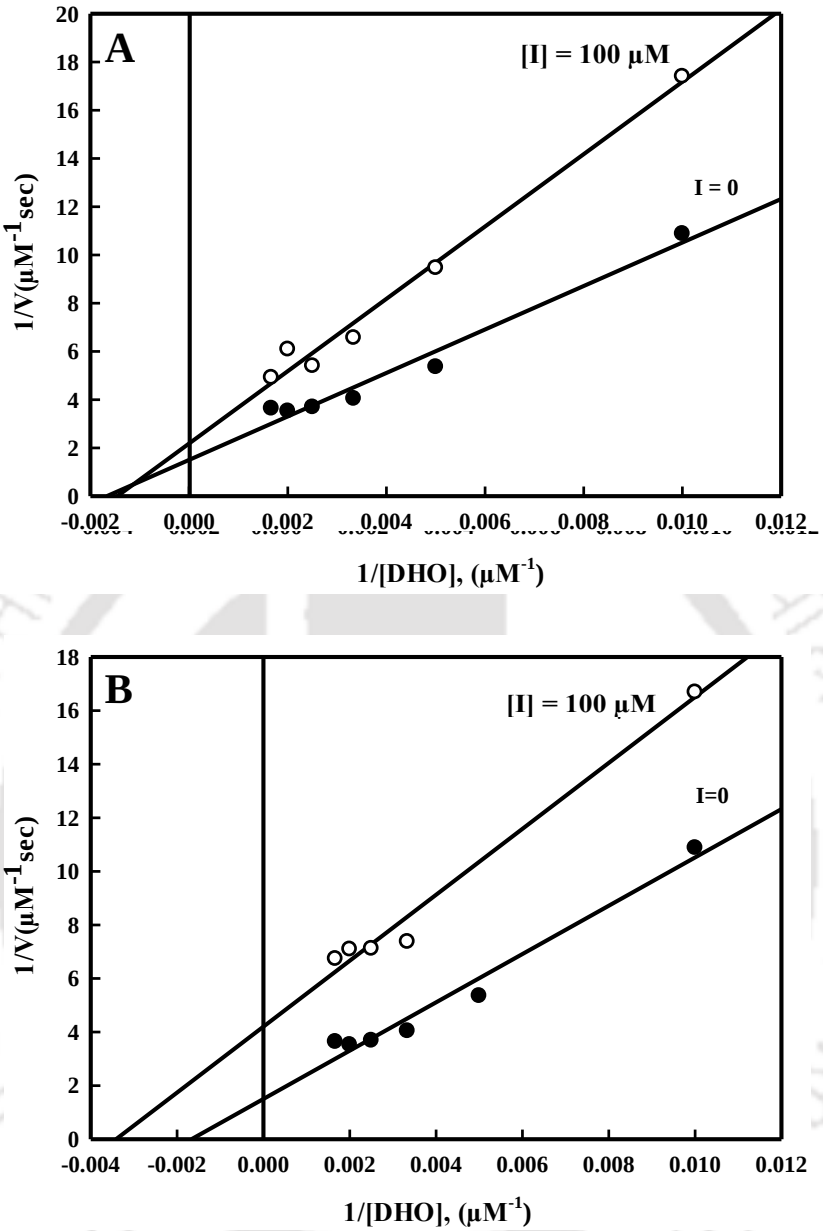


Figure 4.1: Inhibition studies on *L. donovani* dihydroorotase. **(A)** Kaempferol mediated inhibition of dihydrorootase **(B)** Biotin sulfone mediated inhibition of dihydroorotase. Kaempferol displayed a mixed type of inhibition of Ld DHOase while biotin sulfone displayed a uncompetitive inhibition of Ld DHOase. The inhibition constant (K_i) for kaempferol was calculated to be 151.4 µM while that for biotin sulfone was 55.4 µM. DHO: Dihydroorotate.

4.3.2 Anti-leishmanial effects of the inhibitors of *de novo and salvage pathways enzyme(s)*

Kaempferol and biotin sulfone were further assessed for their anti-leishmanial activity **(Figure 4.2A and 4.2B)**. However, there was no substantial parasitic death even at higher concentration of compounds. Parasite survival was also assessed by applying a salvage pathway inhibitor

zebularine **(Figure 4.2C)**, which inhibits cytidine deaminase of salvage pathway *(Lemaire et al., 2009)*. We have tested zebularine alone and in combination with kaempferol (*de novo* pathway inhibitor identified in current study) **(Figure 4.2C)** to assess the parasitic death when both pathways are inhibited. However, significant parasitic death was not observed when treated with zebularine alone, suggesting the reliance on the parallel *de novo* pathway. However, the combination of kaempferol and zebularine shows considerable effect with over ~50% parasitic death **(Figure 4.2C)**.

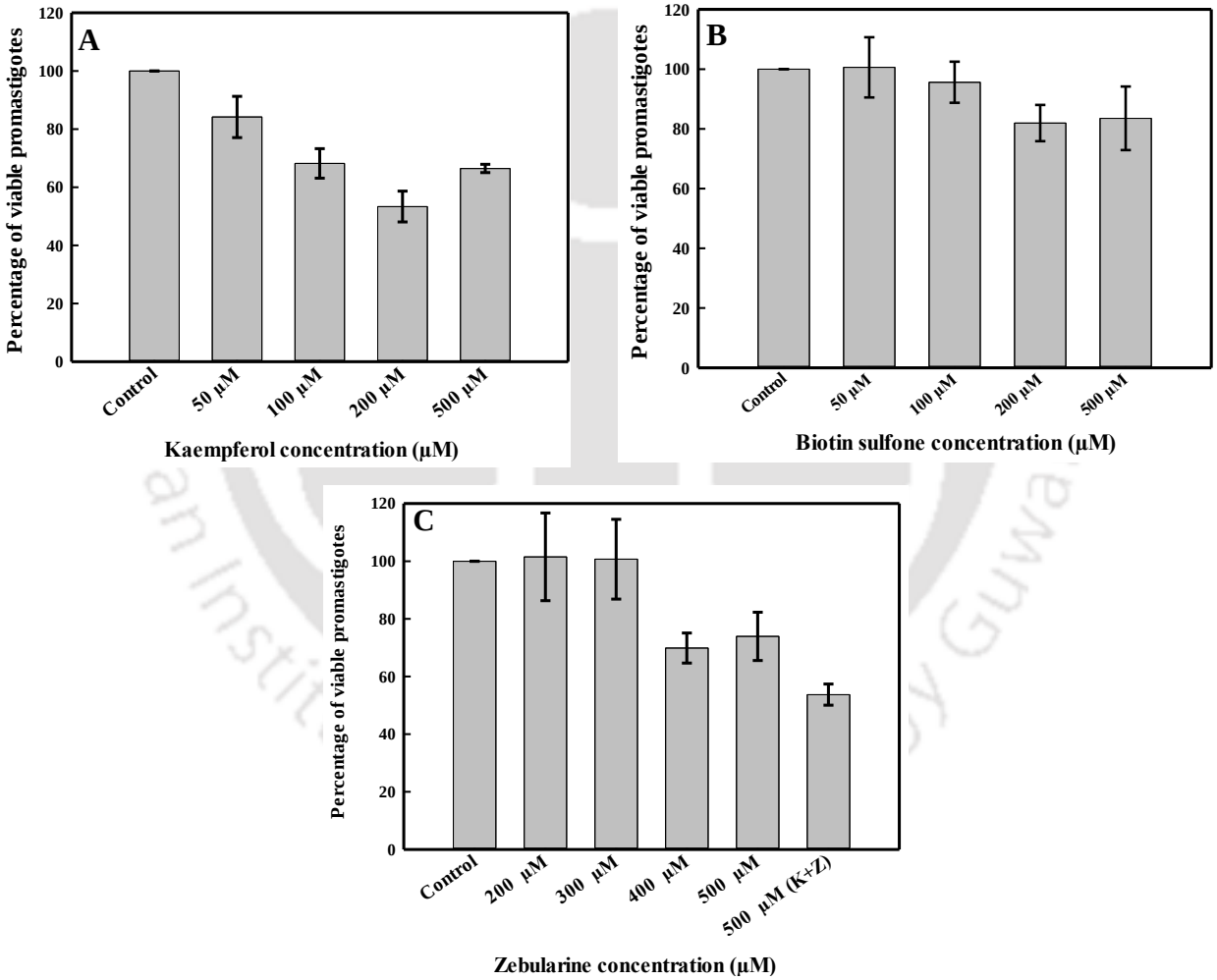


Figure 4.2: Inhibition studies on *L. donovani* promastigotes **(A)** Cell viability assay with kamepferol **(B)** Cell viability assay with biotin sulfone. **(C)** Cell viability assay with zebularine, K+Z is kaempferol and zebularine treatment. Substantial cell death of the parasite was not observed possibly because of the existence of a parallel salvage pathway which circumvented the inhibitory effects of kaempferol and biotin sulfone.

4.3.3 Kaempferol mediated perturbation of pyrimidine pathway

Upon analyzing the cell viability data after treatment of *L. donovani* promastigotes with kaempferol, it was evident that the compounds were unable to induce substantial cell death even at higher concentrations. At this point we speculated that the cell survival was assured by a parallel salvage pathway of pyrimidine biosynthesis as kaempferol was intended to inhibit the *de novo* pathway through DHOase mediated inhibition. Even though kaempferol was inhibiting the *Ld*DHOase but the effect was not evident because of a functional salvage pathway, thus we set to look out whether this was the case. If the above speculation was true than the expression of salvage pathway genes should increase so that same amount of UMP (Uridine monophosphate) is still synthesized in the parasite to combat the inhibitory effect. All salvage pathway genes were found to be overexpressed in the kaempferol treated parasites **(Figure 4.3A)**. Also increased expression of Nucleobase transporter (*NT*) and Nucleoside transporter 1 (*NT1*) were observed **(Figure 4.3A)**, in support of previous data. Even the *de novo* pathway genes underwent significant fold change including DHOase. Further, salvage pathway was inhibited by zebularine to observe how *de novo* pathway genes respond. Interestingly *ATC* (Aspartate transcarbamoylase) and *UPRT* (Uracil phosphoribosyltransferase) underwent some degree of overexpression in zebularine treated parasites **(Figure 4.3B)**. *ATC*, the first regulatory enzyme of *de novo* pathway underwent overexpression in zebularine treated parasites, while *UPRT* which results in the formation of UMP in the final step of pyrimidine salvage pathway and is also a regulatory enzyme *(Villela et al., 2013)* was also overexpressed in order to combat the inhibitory effect of zebularine.

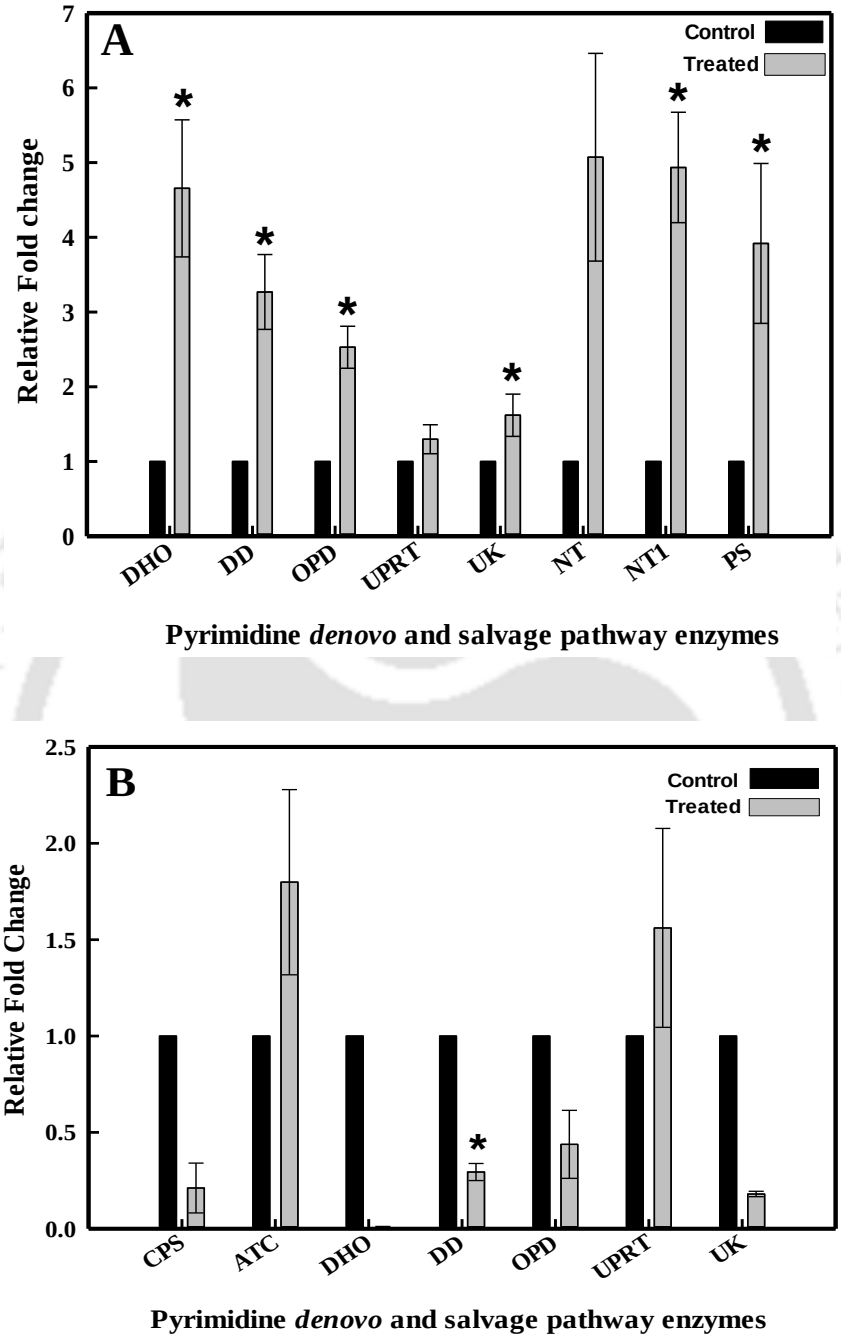


Figure 4.3: **(A)** Expression analysis of salvage and *de novo* pyrimidine biosynthesis pathway genes under kaempferol treatment. (*DHOase*) Dihydrorotase (*DD*) Dihydroorotate dehydrogenase (*OPD*) orotidine-5-phosphate decarboxylase *(UPRT*) Uracil phosphoribosyl transferase (*UK*) Uridine Kinase (*NT*) Nucleobase transporter (*NT1*) Nucleoside transporter 1 (*PS*) tRNA pseudouridine synthetase A. **(B)** Expression analysis of *de novo* and salvage pyrimidine biosynthesis pathway genes under zebularine treatment. (*CPS*) Carbamoyl phosphate synthase (*ATC*) Aspartate transcarbamoylase (*DHO*) Dihydroorotase (*DD*) Dihydroorotate dehydrogenase (*OPD*) orotidine-5-phosphate decarboxylase (*UPRT*) Uracil phosphoribosyl transferase (*UK*) Uridine Kinase. (Statistical significance done by Students's unpaired *t*-test * $p \leq 0.05$)

Table 4.1: Primers used for qRT-PCR

S.No.	Gene	Forward Primer (5'to 3')	Reverse Primer (5'to 3')
1	DHO	CAGAGTGCCATCGACCTG	GGTCGTGAGAAACAGGTGATG
2	UPRT	GCTTCACTTCCTCTTCACTG	ACATTAAACGGCTTCACAGG
3	UK	GCGGAACTGTGTAAAGAGATG	GCTCAATGACAGACTTGAAGG
4	PS	CTTTGACCCTGTCCTTGTG	ACCTTGACCTTGACCGAG
5	NT1	CCTTCCCGACCAAGTTTATG	CGATTCCAATGCCGAAGTAG
6	NT	ACATTCTCACCTTCTACAACG	GAATTACGAACACCTCCACC
7	DHOD	GTTCGACTTCTATCTGGCCTAC	ATTCAGTTCCAGGATGACGC
8	OPRT	AACTTCACGCTCAAGTCAGG	ACGGTCGAACTTGTAATGGC
9	CPS	ATCTCTTTCCCTTCCGTGATG	GGCGGTTAAAGCTAAACATGG
10	ATC	ACAATCGACACCCTGCG	TTCGCCCTTGTTCACAGAC
11	Alpha-tubulin	CTACGGCAAGAAGTCCAAGC	CAATGTCGAGAGAACGACGA

4.3.4 Kaempferol and zebularine induced DNA damage in the L. donovani parasites

As pyrimidine is essential for DNA biosynthesis, we have evaluated the effect of inhibition of *de novo* and salvage pathway inhibition alone and in combination. Control cells grown in normal FBS displayed classical distribution of cell cycle stages while cells maintained in dialyzed FBS exhibited a lesser degree of DNA fragmentation **(Figure 4.4A and 4.4B)**. While a higher proportion of cells displayed DNA fragmentation in kaempferol (26.9 %) **(Figure 4.4C)** and zebularine (20.0%) **(Figure 4.4D)** treated *L. donovani* promastigotes. Combinatorial treatment with both inhibitors resulted in 68.6% **(Figure 4.4E)** of population in DNA damaged region. Noticeably the kaempferol treated population was G1 arrested. Our flow cytometer based data suggests that DNA damage is being induced when single pathway is blocked by their respective inhibitors, also DNA damage is pronounced when both the routes of pyrimidine biosynthesis are blocked by kaempferol and zebularine in the parasite.

Flow cytometer based data clearly represents the DNA damage being done by the inhibitors as the blockage of one pathway at once or both would diminish the pyrimidine supply for nucleic acid synthesis leading to decreased DNA content.

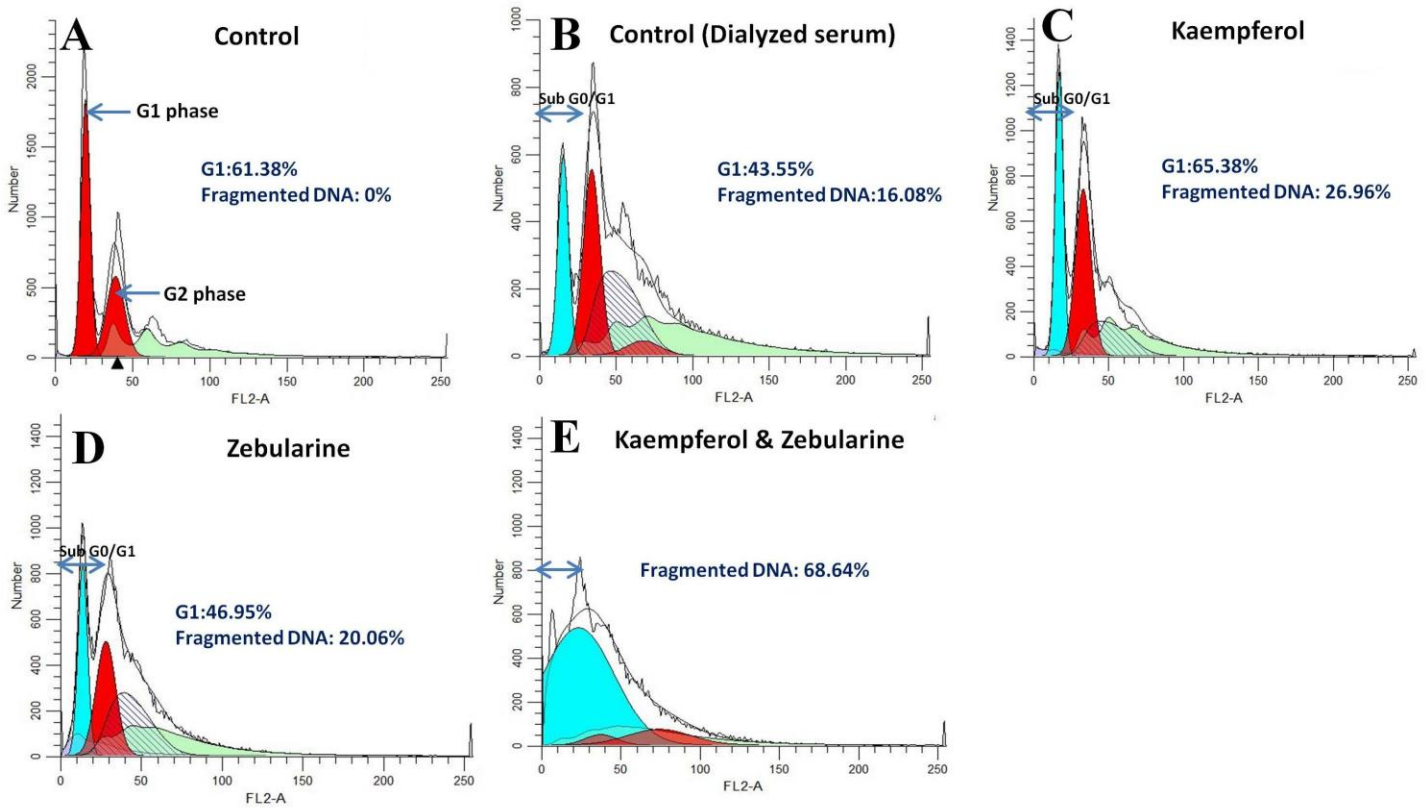


Figure 4.4: DNA content analysis of *L. donovani* in kaempferol and zebularine treated cells **(A)** Cells grown in media supplemented with 15% FBS, **(B)** Cells grown in media supplemented with 15% Dialyzed FBS, **(C)** 500 µM kaempferol treated cells, **(D)** 500 µM zebularine treated cells, **(E)** 500 µM kaempferol+zebularine treated cells. Small molecule inhibitor mediated inhibition of *de novo* pyrimidine biosynthesis pathway should impact the overall nucleotide turnover which would further impact the integrity of DNA which was verified by flow cytometer based analysis which revealed that blocking single pathway (*de novo* or salvage) simultaneously had lesser impact on genomic integrity then the blockage of both pathways. The flow cytometry based studies furthered our previous conclusions that both *de novo* and salvage pathways play a vital role in the growth and survival of the parasite.

4.4 Discussion

Pyrimidine pathways of *L. donovani* were explored by studies on Δ*cps* (carbamoyl phosphate synthetase) null mutants and inhibition studies on UPRT (uracil phosphoribosyltransferase) enzyme and subsequent effect on the parasite *(Wilson et al., 2012).* The Δ*cps* phenotype rendered parasites in an attenuated state resulting in reduced parasite burdens while substrate inhibition of UPRT by uracil resulted in increased growth sensitivity of *L. donovani* pyrimidine auxotrophs *(Wilson et al., 2012).* Although kaempferol and biotin sulfone both exerts their inhibitory effects on recombinant DHOase but the parasite evades the *de novo* pathway inhibition by overexpressing salvage pathway genes in an attempt to form same amount of UMP (Uridine monophosphate), the common end product of both pathways. We also observed the increased expression of nucleobase and nucleoside transporters. Similarly, *de novo* pathway regulatory genes like *ATC* (Aspartate transcarbamoylase) etc. are up-regulated when the salvage pathway is inhibited by zebularine. The data supports the fact that either of the two pathways can support the growth of the parasite. Both *de novo* and salvage pathways culminate in the synthesis of UMP which is further processed to form UDP and UTP respectively. Inhibition of the *de novo* pathway would lead to the overexpression of salvage pathway genes so as to ensure that same amount of UMP is still synthesized. Indeed it was the case as evident by qRT-PCR data. Genes involved in salvage pathway like *UPRT* and *UK* were overexpressed in the kaempferol treated parasites. Notably Nucleobase transporter (*NT*) Nucleoside transporter 1 (*NT1*) involved in the nucleotide transport in the parasite also underwent significant fold change as the parasite in the presence of inhibitor will enhance the uptake of preformed nucleotides and nucleobases from the external environment so as to ensure substantial UMP synthesis. Under inhibition conditions both *de novo* and salvage pathway genes underwent significant fold changes but when compared salvage pathway genes like *PS* and specifically transporters like *NT* and *NT1* underwent higher fold change as compared to *de novo* pathway genes like *DD*, *OPD* etc. The plausible reason of higher fold expression of *de novo* pathway genes may be attributed to the limited substrate availability to Dihydroorotate dehydrogenase (*DD*) as lesser amounts of Dihydroorotate would be formed when dihydrorootase is inhibited causing *DD* to increase its expression so as to make almost equal amounts of end product, similar is the case with *OPD* which would get lesser substrate as compared to uninhibited conditions leading to its overexpression. While manifold expression of salvage pathway genes, even higher than *de novo* pathway genes is easily evident

as it is a parallel pathway for *de novo* pathway and should express highly in order to cope up with the inhibitory effects on *de novo* pathway and make equal amounts of UMP. Both pathways need to be inhibited so as to ensure proper cell death.

All the above mentioned results indicate that when inhibitor(s) of the *de novo* and salvage pathway are applied independently, they do not result in cell death while when applied in combination they lead to ~50% parasitic death. This signifies that the parasite relies on a parallel salvage pathway which circumvents the inhibitory effect of *de novo* pathway inhibitors by altering their gene expressions. Similarly, when the salvage pathway is inhibited the *de novo* pathway alters its gene expressions to overcome the inhibitory effects. Also when both pyrimidine pathways are inhibited independently they show very less DNA fragmentation while pronounced DNA fragmentation was observed when both pathways are inhibited. Similar, observation was reported on *Trypanosoma brucei (Ali et al., 2013)*. The data clearly points out that the inhibition of single pathway can be overcome by increased expression of enzyme(s) of alternate pathway and both pathways are equally important in the pathogen. Thus, inhibition of both pathways of pyrimidine biosynthesis could be a good strategy of novel drug development. It is worth mentioning that zebularine also inhibits DNA methyl transfereases *(Zhou et al., 2002)* and given that the aromatic structure of kaempferol, one could expect some intercalating effect on the DNA. Contribution of these effects on DNA damage and parasitic death cannot be ruled out.

4.5 Conclusion

Mixed and uncompetitive inhibition of *Ld* DHOase by kaempferol and biotin sulfone was unable to show significant cell death of the parasite suggesting the recovery being done by the salvage pathway. Kaempferol and zebularine mediated inhibition of *de novo* and salvage pathway led to an alteration in the gene expression of pyrimidine pathway, and significant DNA damage was also being induced. In presence of these inhibitors alone or in combination, we observed the altered mRNA expression level of *de novo* and salvage pathway enzymes and decrease in DNA content suggesting their physiological significance in cell viability. A parallel salvage pathway prevented the inhibitory role of the mentioned inhibitors which was also physiologically implied in terms of decrease in DNA content.

CHAPTER 5

Subcellular localization studies on *L. donovani* asparaginase variants*

ABSTRACT

Asparaginase, a pivotal enzyme of the aspartate metabolic pathway is present in the form of two variants in the *Leishmania donovani* parasite which is the causative agent of visceral leishmaniasis (VL). Aspartate pathway is closely linked to the *de novo* pyrimidine pathway as it provides precursors in the form of carbamoyl aspartate (dihydroorotase substrate) to the *de novo* pyrimidine pathway. The growing concern in the scientific community regarding drug resistant strains of *Leishmania* is providing a fresh impetus to investigate metabolic pathways of the parasite. Aspartate metabolic pathway is one such interesting pathway which is not adequately studied and needs further attention. Whether these asparaginase variants confer any advantage in the growth or infectivity of the parasite remains to be addressed. The current study using a combination of *in silico* and experimental approaches deduced out the cytosolic localization of both variants of asparaginase in the *L. donovani* parasite. This further widens up many interesting implications on the functional aspects of asparaginases. Further studies will reveal the functional aspects of the two variants of asparaginases in the *L. donovani* parasite.

*** Part of the work is submitted for publication**

5.1 Introduction

Leishmania donovani is the causative agent of visceral leishmanisis (VL) a neglected tropical disease prevalent mostly in the third world countries. Female sandfly known as *Phlebotomus* (in the old world) and *Lutzomyia* (in the new world) is mainly responsible for the spread of this disease as the former is the carrier of the parasite. Emerging drug resistance for VL has been a major concern in the recent years prompting investigators to relook into the metabolic pathway of the parasite and to come up with novel drug targets.

Asparaginases have been classified into two classes based on their presence. Type I asparaginases are predominantly localized in the cytosol of *E. coli* and other species while Type II asparaginases are secreted out. Type II asparaginases have been utilized in the treatment of acute lymphoblastic leukemia (ALL). Asparaginase (EC: 3.5.1.1) is a key enzyme of the aspartate metabolic pathway which leads to the conversion of asparagine into aspartate. The aspartate thus formed is reshuffled into many other vital metabolic pathways like Krebs cycle and *de novo* pyrimidine biosynthesis pathway. In the Krebs cycle it is supplied in the form of fumarate and oxaloacetate while in the *de novo* pyrimidine biosynthetic pathway it is supplied in the form of N-carbamoyl aspartate which is a dihydroorotase substrate, the third enzyme of the *de novo* pyrimidine biosynthetic pathway. Although aspartate is also formed from oxaloacetate via aspartate aminotransferase enzyme, but which route of aspartate formation predominates especially in the parasite such as *L. donovani* remains to be elucidated. Thus the presence of asparaginase is an added advantage as the aspartate is anyhow formed via aspartate aminotransferase thus this gives rise to the possibility of the other roles played by asparaginase apart from growth of the parasite. In a study asparagine has also been shown to be involved in the autophagy process in *L. donovani (Stephanie et al., 2006)*. If anyhow it contributes somehow in the infectivity of the parasite then it can be validated as a drug target which will help in the development of treatment regimens for visceral leishmaniasis.

5.2 Materials and methods

5.2.1 Materials

Leishmania donovani strain BHU 1081 was a kind donation from Prof. Shyam Sundar, Banaras Hindu University, India. The culture conditions for *L. donovani* are well established in our laboratory *(Saudagar et al., 2013, Das et al., 2015)*. In brief, the *L. donovani* was grown in

M199 medium supplemented with 15% heat inactivated fetal bovine serum (FBS), 100Uml^{-1} and 100 µgml^{-1} streptomycin. pGL vector used for localization studies carrying GFP ORF (Green fluorescent protein) was a kind gift from Prof. David Mottram (University of Glasgow, Scotland, UK). The DreamTaq Green DNA polymerase was purchased from Thermo scientific. All restriction enzymes were purchased from ILS (NEB). All other chemicals were of highest purity grade, procured from Sigma Aldrich or Merck. Hoechst 33342 was from invitrogen and anti fade reagents were from Sigma Aldrich.

5.2.2 Subcellular localization prediction using in silico approaches

Various online available tools like Cello v 2.5 (http://cello.life.nctu.edu.tw) *(Yu et al., 2006)* were employed to predict the localization of *L. donovani* asparaginase. Homology based search using NCBI BLAST was also employed to assess the subcellular localization of *L. donovani* asparaginase variants.

5.2.3 Cloning of L. donovani asparaginase variants in pGL vector

1197 bp ORF of *L. donovani* cytoplasmic asparaginase like protein (LDBPK_150440) was PCR amplified using gene specific primers (FP 5' GAAGATCTATGGAAGCGGGAATAGAGG 3', RP 5' CCGCTCGAGTCACAGCTTCGCATG 3'). The amplicon and pGL vector were double digested with *Bgl*II and *Xho*I and ligated at 25°C, 2 hrs. Ligated product was transformed into competent *E. coli* DH5alpha cells using heat shock method. The recombinant clones thus obtained were confirmed by double digestion and sequencing. 1110 bp ORF of *L. donovani* asparaginase like protein (LDBPK_364650) was PCR amplified using gene specific primers (FP 5' GGCATGGACGAGCTGTACAAGAGATCTATGCTGTACATCAGTAGCGGCC 3', RP 5' GTGGTGGTGGTGGTGGTGCTCGAGTCACAGCTTCGCGGACGGCG 3'). pGL vector was double digested with *Bgl*II and *Xho*I. Amplicon was ligated to digested pGL vector using Gibson assembly protocol *(Gibson et al., 2009)*. Ligated product was transformed into competent *E. coli* DH5alpha cells using heat shock method. Positive clones were confirmed as stated above by digestion and sequencing.

5.2.4 Generation of L. donovani cells expressing GFP tagged asparaginase variants

Leishmania donovani promastigotes grown up to log phase (10^6 cells) were electroporated with pGL vector carrying a fusion of GFP and *L. donovani* asparaginase variants using protocol as established in our lab. Briefly 10^6 cells were pelleted down at 1000 rcf, 6 min and washed twice with PBSG (10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 145 mM NaCl and 2% glucose). Pellet was further resuspended in Electroporation Buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and pGL fusion construct was added to the cell suspension and kept in ice for 10 mins. After 10 mins cells were electroporated using Gene Pulsar Xcell™ Electroporation system (Biorad) exponential protocol (450 V, 400 µF capacitance, 50 Ω resistance for 2mm cuvette). Cells were also mock electroporated as controls. Following electroporation cells were kept in ice for 10 minutes and were resuspended in M199 media and kept at 25°C for one day. The positive cells were selected in the presence of 20 µg G418 antibiotic as the pGL vectors carries neomycin as the eukaryotic selection marker. The selected cells were further used for green fluorescence visualization.

5.2.5 Cellular imaging using inverted microscope

G418 positive *L. donovani* promastigotes were visualized for the green fluorescence in Nikon inverted microscope (Eclipse Ti U). The parasites were further stained with Hoechst 33342 which stains nucleus. In brief cells were pelleted down and washed twice with PBS (Phosphate Buffered Saline, pH 7.4) to remove traces of medium. Cells were fixed by resuspending them in 4% paraformaldehyde for 30 minutes, 25°C. Fixed cells were then washed thrice with PBS to remove the paraformaldehyde. Cells were permeabilized by 0.1% Triton X 100 for 10 min, 25°C. Cells were again washed twice with PBS and counterstained with Hoechst 33342 (1 µM) for 3-5 minutes and further washed twice with PBS. Cells were mounted using the anti-fade reagent (propyl gallol). Cells were imaged using a Nikon inverted microscope (Eclipse Ti U) at 100X oil immersion objective with excitation wavelengths of 488 nm and 350 nm for GFP and Hoechst 33342 respectively.

5.3 Results

5.3.1 Subcellular localization of L. donovani asparaginase variants using in silico approaches

Leishmania donovani genome codes for two variants of asparaginase: cytoplasmic asparaginase like protein (LDBPK_150440) on chromosome no. 15 and asparaginase like protein (LDBPK_364650) on chromosome no. 36. Cytoplasmic asparaginase like protein has been annotated as cytosolic which determines its localization like other asparaginases but the localization status of asparaginase like protein has not been designated. *E. coli* codes for two types of asparaginases: Type I asparaginase which is cytosolic and Type II asparaginase which is secreted out. Firstly we sought to determine whether the *L. donovani* asparaginases belong to different types or they fall in the same category. Based on homology based analysis using NCBI BLAST it was revealed that the two asparaginase variants of *L. donovani* belonged to Type I class. Although Type I asparaginases are localized predominantly in the cytosol still we sought to determine the localization of both variants of asparaginases in the *L. donovani* parasite. The localization patterns of both variants of *L. donovani* asparaginase were determined using *in silico* and experimental approaches. Cello v 2.5 based online server was used to determine the cellular localization of *L. donovani* asparaginase variants. *In silico* predictions point out a cytosolic localization for both *L. donovani* asparaginase variants **(Figure 5.1)**.

CELLO RESULTS

SeqID: CBZ32861.1 cytoplasmic l-asparaginase i-like protein [Leishmania donovani]

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Cytoplasmic	0.950
N-peptide Comp.	Cytoplasmic	0.580
Partitioned seq. Comp.	Cytoplasmic	0.943
Physico-chemical Comp.	Cytoplasmic	0.716
Neighboring seq. Comp.	Cytoplasmic	0.611

CELLO Prediction:

	Cytoplasmic	3.800 *
	Periplasmic	0.617
	InnerMembrane	0.523
	OuterMembrane	0.048
	Extracellular	0.013

****

CELLO RESULTS

SeqID: CBZ38902.1 asparaginase-like protein [Leishmania donovani]

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Cytoplasmic	0.921
N-peptide Comp.	InnerMembrane	0.687
Partitioned seq. Comp.	Cytoplasmic	0.961
Physico-chemical Comp.	Cytoplasmic	0.924
Neighboring seq. Comp.	Cytoplasmic	0.908

CELLO Prediction:

	Cytoplasmic	3.966 *
	InnerMembrane	0.911
	Periplasmic	0.084
	OuterMembrane	0.024
	Extracellular	0.014

****

Figure 5.1: Cello v 2.5 based predictions for the subcellular localization of *L. donovani* cytoplasmic asparaginase like protein (right) and asparaginase like protein (left).

5.3.2 Cloning of L. donovani asparaginase variants in pGL GFP vector

Leishmania donovani asparaginase variants were cloned in pGL vector downstream of GFP tag (Green Fluorescent Protein). The putative constructs were confirmed by double digestion and sequencing **(Figure 5.2A and B)**.

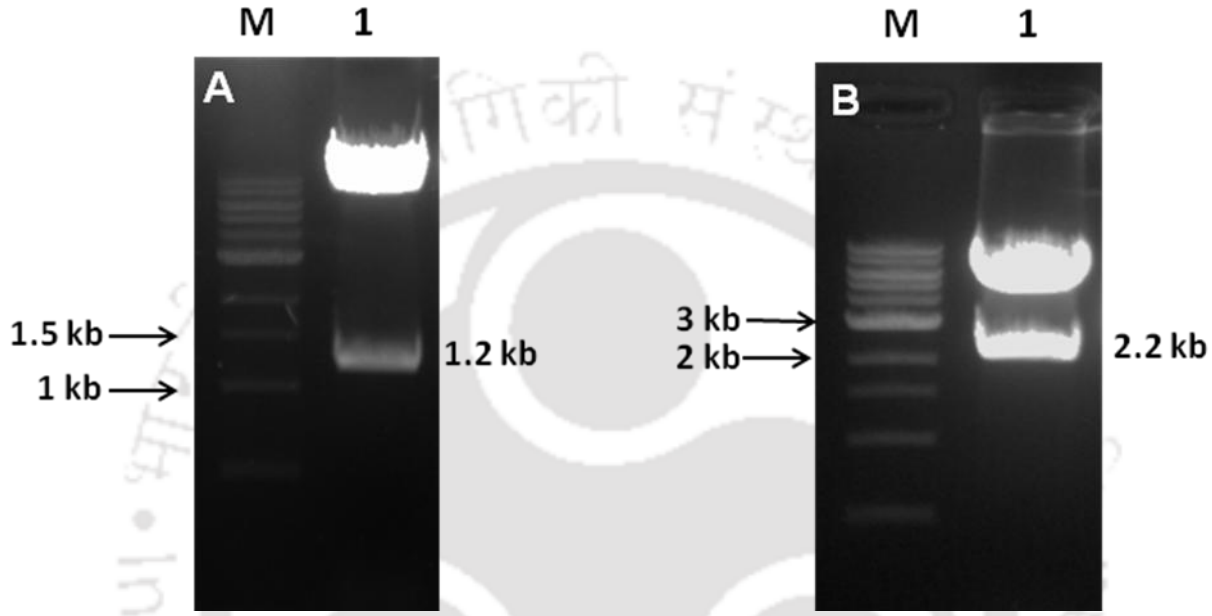


Figure 5.2: Cloning of *L. donovani* asparaginase variants in pGL GFP vector. **(A)** Double digestion of pGL GFP *Ld* AnsA (LDBPK_150440) vector with *Bgl*II and *Xho*I showing a release of 1.2 kb. **(B)** Double digestion of pGL GFP *Ld* AnsA (LDBPK_364650) vector with *BstE*II and *Nde*I showing a release of 2.2 kb.

5.3.3 Subcellular localization of L. donovani asparaginase variants using microscopy

Subcellular localization of *L. donovani* asparaginase variants was determined by visualizing the GFP fluorescence in Nikon Eclipse Ti microscope, also the cells were counter stained with Hoechst 33342 to stain the nucleus so that localization can be determined in a distinct way. Fluorescence images for blue and green channels were retrieved and merged with DIC image to generate a merged image to show the localization of asparaginase variants. Microscopic examination revealed that both cytoplasmic asparaginase like protein and asparaginase like protein are localized in the cytosol of *L. donovani* parasite **(Figure 5.3 and 5.4)**.

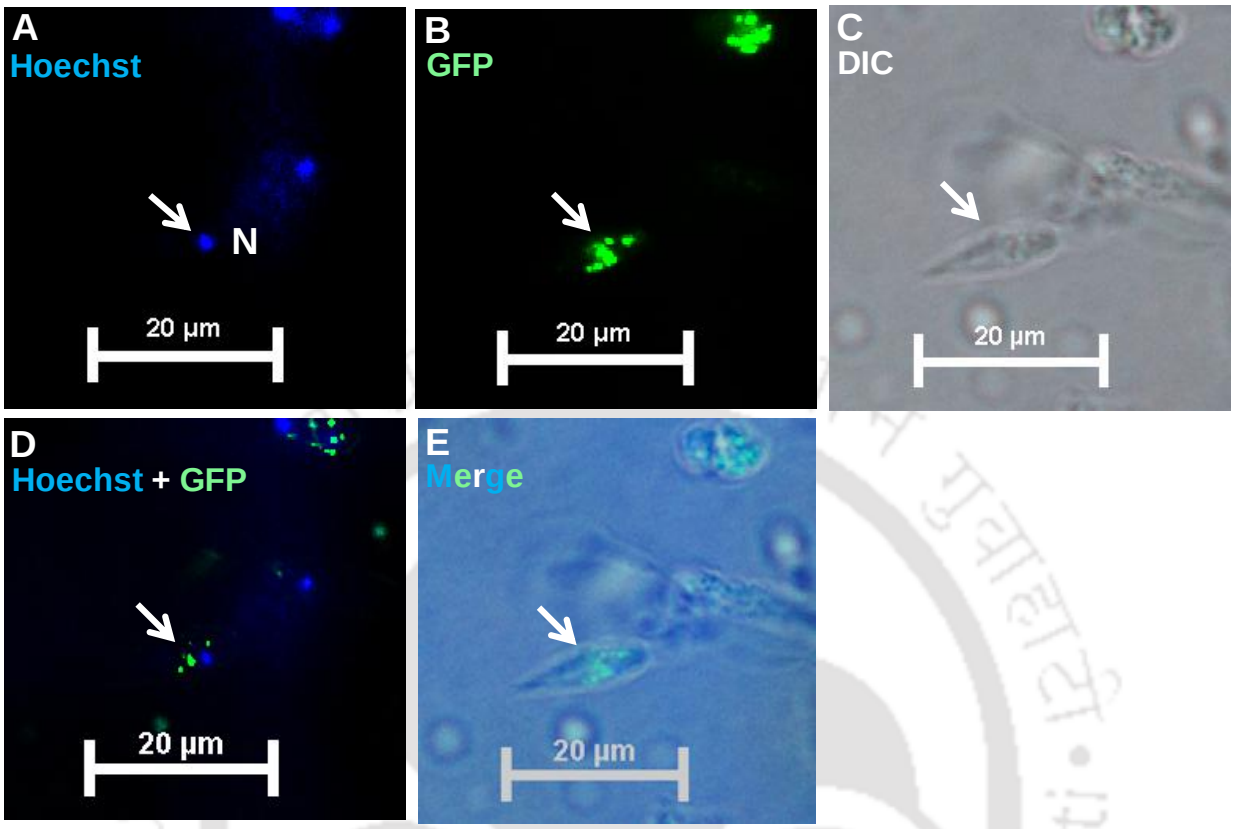


Figure 5.3: Subcellular localization of *L. donovani* cytoplasmic asparaginase like protein (LDBPK_150440). **(A)** *Leishmania donovani* nucleus stained with Hoechst 33342 stain designated as N (blue puncta). **(B)** Green fluorescence from cytoplasmic asparaginase like protein (LDBPK_150440) in the cytosol. **(C)** DIC (Differential Interference Contrast) bright field image of *L. donavani* parasite. **(D)** Overlay of images A and B to show distinct localization of cytoplasmic asparaginase like protein outside of nucleus in the cytosol. **(E)** Overlay of images A, B and C. *Leishmania donovani* cytoplasmic asparaginase like protein (LDBPK_150440) is distinctly localized in the cytosol of the parasite.

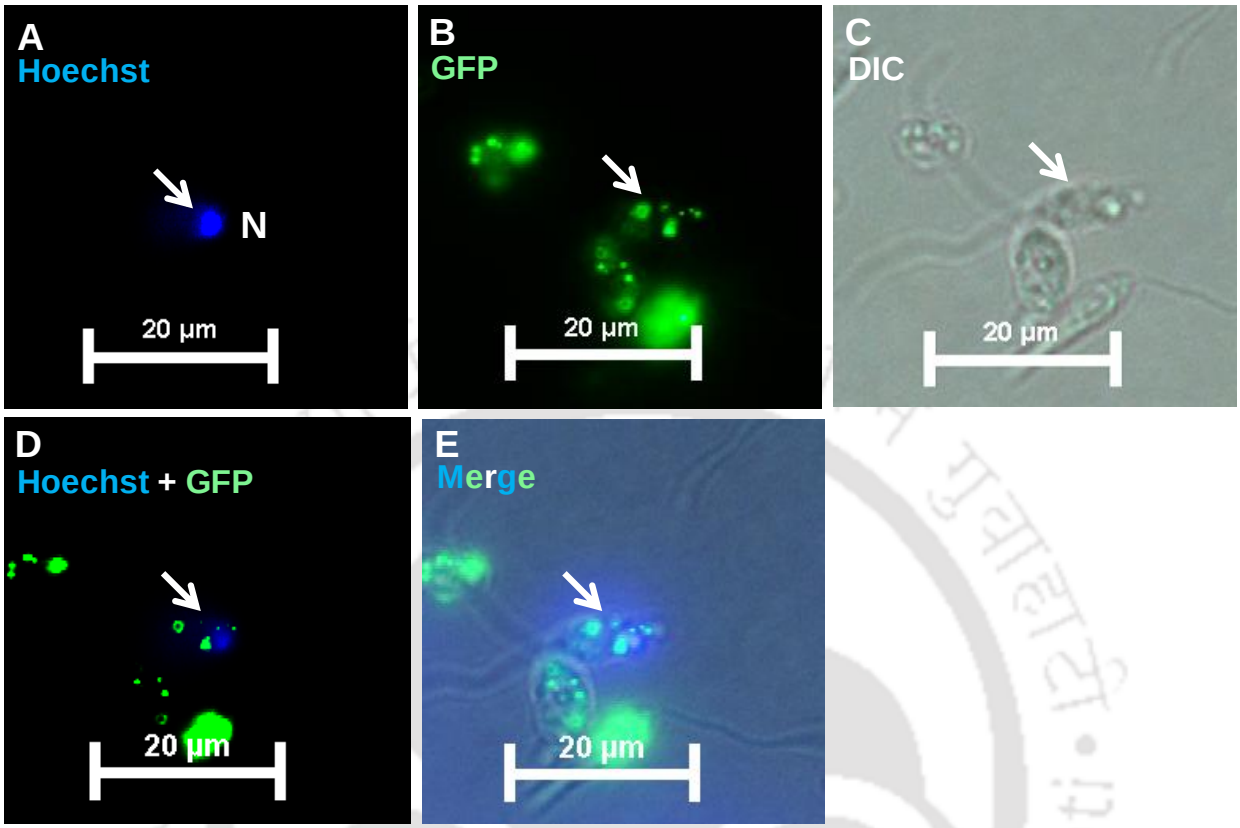


Figure 5.4: Subcellular localization of *L. donovani* asparaginase like protein (LDBPK_364650) **(A)** *Leishmania donovani* nucleus stained with Hoechst 33342 stain designated as N (blue puncta). **(B)** Green fluorescence from asparaginase like protein (LDBPK_364650) in the cytosol. **(C)** DIC (Differential Interference Contrast) bright field image of *L. donavani* parasite. **(D)** Overlay of images A and B to show distinct localization of asparaginase like protein outside of nucleus in the cytosol. **(E)** Overlay of images A, B and C. *Leishmania donovani* cytoplasmic asparaginase like protein (LDBPK_364650) is distinctly localized in the cytosol of the parasite.

5.4 Discussion

All *Leishmania* species possess two variants of Type I asparaginase while many other parasitic species do not possess asparaginase at all which points out to the specific presence of asparaginase in the *Leishmania* parasite which may confer some advantage in terms of infectivity and growth which needs to be elucidated. All trypanosomatid species except *Trypanosome cruzi* lacks asparaginase and even in *T. cruzi* two variants of asparaginase are present which are very

close to the *L. donovani* variants. Another related trypanosomatid *Crithidia acanthocephali* has asparaginase enzyme while many other significant parasitic species like *Plasmodium* and *Toxoplasma* lack the asparaginase enzyme. *Mycobacterium tuberculosis* is another intracellular pathogen like *L. donovani* which resides in the phagosome of macrophages in the acidic environment. *Mycobacterium tuberculosis* secretes asparaginase extracellularly which has been shown to neutralize the acidic environment of phagosome thus aiding in its survival *(Gouzy et al., 2014)*. Type II asparaginase of *Salmonella typhimurium* also plays key role in enhancing the infectivity of the pathogen *(McLaughlin et al., 2016)*. Thus the presence of asparaginase variants in the *L. donovani* parasite makes up interesting possibilities of their contributions in the infectivity of the parasite.

In the present study a combination of *in silico* and experimental approaches gave an insight that the two variants of *L. donovani* asparaginase are localized in the cytosol. This finding leads to the possibilities of many interesting outcomes that may or may not decide the advantage conferred on *L. donovani* by these asparaginase variants. Either both or one of these variants may be active in the parasite in case if they exist as independent enzymes. Or the existence of an asparaginase heterodimer cannot be ruled out. Whatever may be the case the essentiality of the asparaginase variants in the parasites will be ruled out by genetic and biochemical studies. As the human counterpart lacks the asparaginase enzyme this makes the *L. donovani* asparaginase a vulnerable and useful target that can be utilized for therapeutic interventions. Further studies will shed more light on the unknown contributions of asparaginase variants in the *L. donovani* parasite.

5.5 Conclusion

In the present study the subcellular localization of *Leishmania donovani* asparaginase variants was studied. *Leishmania donovani* asparaginase variants tagged with GFP were found to be localized in the cytosol of the parasite. Experimental data was also supported by *in silico* findings which also revealed the cytosolic presence of *L. donovani* asparaginase variants. Further studies on the *L. donovani* asparaginase variants will reveal their role in the growth and infectivity of the parasite.

CHAPTER 6

Summary

ABSTRACT

Dihydroorotase, the third enzyme of the *de novo* pyrimidine biosynthesis pathway of *Leishmania donovani* was biochemically characterized in both directions which revealed a preference for biologically significant forward direction reaction. Morover, the forward reaction was preferred in slightly acidic pH while slightly alkaline pH favored reverse reaction. A tetrameric structure was exhibited by dihydroorotase. Docking studies revealed biotin sulfone and kaempferol as potential inhibitors of dihydroorotase which were later found to inhibit the recombinant enzyme in a mixed and uncompetitive manner, respectively. Dihydroorotase inhibitor could not elicit significant parasitic death as the same was prevented by a parallel salvage pathway. The over expression of salvage pathway enzymes was demonstrated by qRT PCR and supported by DNA degradation assay. Overall these inhibitors have led us to a better understanding of pyrimidine metabolism in the parasite as a whole. The localization patterns of variants of asparaginase, a key enzyme of the aspartate metabolic pathway which provides precursors to the *de novo* pyrimidine pathway were found out to be cytosolic in the *L. donovani* parasite. An understanding of the

pyrimidine and its related aspartate metabolic pathway will aid in the development of therapeutics for the treatment of visceral leishmaniasis.

6.1 Biochemical characterization of *L. donovani* dihydroorotase

Emergence of resistant strains of *L. donovani* in the recent years along with the toxicity of existing treatments has slowed the eradication of visceral leishmaniasis caused by *L. donovani.* Introspection of various pathways has revealed a huge number of drug targets which are still being tested. Drug discovery in combination with effective vaccines promises to decimate visceral leishmaniasis. Rapid advances have been made in these directions with the sequencing of *L. donovani* genome and advancement of techniques. Efficient drug delivery with minimal toxicity is the aim of the eradication programme. Progressing in this direction we compared the *de novo* pyrimidine pathway of the parasite and host and found significant differences in terms of structure and arrangement. Among the six enzymes of the *de novo* pyrimidine pathway the third enzyme dihydroorotase was found to be structurally distinct from the other members of the pathway and was chosen for further studies. Recombinant *L. donovani* dihydroorotase was biochemically characterized to assess its kinetic parameters. Dihydroorotase enzyme catalyzes the ring cyclization reaction leading to the formation of dihydroorotase from carbamoyl aspartate, the reaction being reversible. The K_m and V_{max} for the forward reaction were found out to be 28.1 ± 6.5 μM and 1.2 ± 0.06 μMs^{-1} respectively, while the K_m and V_{max} for the reverse reaction were found out to be 220.75 ± 15.3 μM and 0.404 ± 0.012 μMs^{-1} respectively. The low K_m and higher V_{max} of the forward reaction indicates a preference for the forward direction over reverse direction which is also biologically significant. Existence of the parasite in the dual host system (sandfly and mammalian host) in varying pH (alkaline in sandfly and acidic in the phagolysosome of macrophage) led us to determine the pH optima for dihydroorotase in both the directions. pH optima for the forward direction was estimated to be 6.0 and that for reverse direction was 8.0. The ring cyclization reaction proceeds at a slightly acidic pH while the ring cleavage reaction proceeds at a slightly alkaline pH. Thus precedence for forward reaction was shown at a slightly acidic pH which may have biological implications aiding in the survival in phagolysosomes of macrophages. *Leishmania donovani* dihydroorotase was also shown to be tetramer by size exclusion chromatography. Dihydroorotase from *E. coli* has been reported to be

a homodimer, while the mammalian dihydroorotase forms a part of the multi subunit enzyme referred to as CAD.

6.2 Inhibition studies on *L. donovani* dihydroorotase

Structural distinctness of the parasitic dihydroorotase from the mammalian counterpart led us to look for compounds which could act as potent inhibitors of *L. donovani* dihydroorotase. Along with the antileishmanial effects of these potent inhibitors these inhibitors were also used to compare the relative contributions of *de novo* and salvage pathway of pyrimidine biosynthesis in the protozoan parasite. Since the crystal structure of *L. donovani* dihydroorotase has not been reported yet, thus a template based modeled structure was made using modeler which was further used for docking studies. Structural analog based screening revealed a few inhibitors which could be tested as potent antileishmanials. Among the screened analogs kaempferol and biotin sulfone were further tested for their inhibitory effects. Kaempferol inhibited *Ld* dihydroorotase in a mixed inhibition with a K_i of 151.4 μM while biotin sulfone inhibited *Ld* dihydroorotase in an uncompetitive manner with a K_i of 55.4 μM. These compounds were further assessed for their antileishmanial effects. However application of these compounds was not able to cause substantial parasitic death. At this point it was speculated that a parallel salvage pathway was able to overcome the inhibitory effects of the above mentioned inhibitors and was able to synthesize ample pyrimidines which rescued the parasites.

6.3 Comparative analysis of *de novo* and salvage pathways

Since the salvage pathway was able to overcome the inhibitory effects of the applied *de novo* pathway inhibitors it was thought that the parallel salvage pathway rescued the dying parasites, thus gene expression analysis of both pathways under inhibitory conditions was ensued to further compare the essentiality of both pathways in parasitic growth. Kaempferol mediated inhibition of *de novo* pathway enhanced the expression levels of salvage pathway genes confirming the counteracting effect of the salvage pathway as evident from cell viability assay. To ensure if the same was the case with *de novo* pathway genes, the salvage pathway was inhibited by zebularine (a cytidine deaminase inhibitor) and the expression levels of *de novo* pathway genes was assessed which showed enhanced expression of *de novo* pathway genes. These studies point out the essentiality of both pathways and also the other pathway gets active when the other one is

shut down. The effect of these inhibitors was further assessed by analysing the DNA degradation using flow cytometry. Substantial DNA degradation (68.6%) was observed when both pyrimidine pathways were inhibited by kaempferol and zebularine. While a lesser degree of DNA fragmentation was observed when pathways were inhibited separately. These observations further add to the previous findings that both pathways are equally essential for parasitic growth and survival.

6.4 Subcellular localization of *L. donovani* asparaginase variants

Leishmania donovani genome encodes two variants of asparaginase which belong to Type I category based on amino acid sequence homology and Cello v 2.5 based predictions. Asparaginases in many intracellular parasites like *M. tuberculosis* and *S. typhimurium* has been implicated to exert their effects in the infectivity of the parasites. These findings have raised interest in *L. donovani* asparaginases as what role they have to play in the growth and infectivity of the parasite. To seek preliminary hints we sought to determine the localization status of both variants of *L. donovani* asparaginase. Microscopic studies using GFP tagged asparaginase variants revealed a cytosolic presence of both variants of asparaginase. Experimental findings were also validated using *in silico* approaches like Cello v 2.5 which also predicted a cytosolic localization for *L. donovani* asparaginase variants. Furthermore genetic and biochemical studies will reveal the functional aspects of asparaginase variants of *L. donovani* parasite. The dissertation work has also been pictorially represented in the **Figure 6.1**

Summary of the thesis work

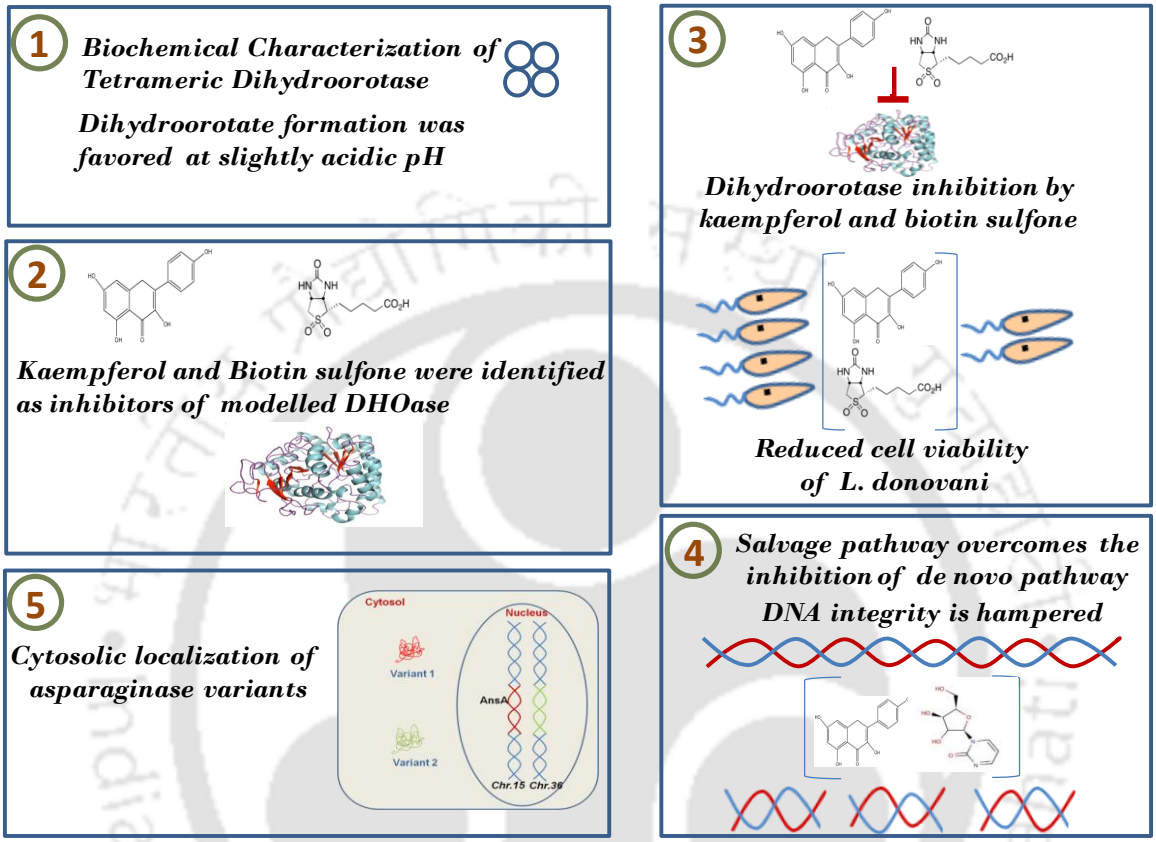


Figure 6.1: Pictorial summary of the thesis work.

Bibliography

Afrin F, Ali N. (1997) Adjuvanticity and protective immunity elicited by *Leishmania donovani* antigens encapsulated in positively charged liposomes. *Infect.Immun.* 65, 2371–2377.

Afrin F, Anam K, Ali N. (2000) Induction of partial protection against *Leishmania donovani* by promastigote antigens in negatively charged liposomes. *J. Parasitol.* 86, 730–735.

Afrin F, Rajesh R, Anam K, Gopinath M, Pal S, Ali N. (2002) Characterization of *Leishmania donovani* antigens encapsulated in liposomes that induce protective immunity in BALB/c mice. *Infect.Immun.* 70, 6697–6706.

Alexander J, Satoskar AR, Russell DG. (1999) *Leishmania species: Models of intracellular parasitism. J. Cell Sci.* 112, 2993–3002.

Ali JA, Tagoe DN, Munday JC, Donachie A, Morrison LJ, de Koning HP. (2013) Pyrimidine biosynthesis is not an essential function for *Trypanosoma brucei* bloodstream forms. *PLoS One.* 8:e58034.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.

Alvar J, Velez ID, Bern C, Herrero M, Desjeux P Cano J, Jannin J, den Boer M. (2012) Leishmaniasis Worldwide and Global Estimates of Its Incidence. *Plos One*. 7: e35671. doi: 10.1371/journal.pone.0035671.

Andersson Rasmussen A, Kandasamy D, Beck H, Crosby SD, Björnberg O, Schnackerz KD, Piškur J. (2014) Global expression analysis of the yeast Lachancea (Saccharomyces) kluyveri reveals new URC genes involved in pyrimidine catabolism. *Eukaryot Cell*. 13, 31–42.

Ashutosh, Sundar S, Goyal N. (2007) Molecular mechanisms of antimony resistance in *Leishmania*. *J Med Microbiol*.56, 143–153.

Bardeleben C, Sharma S, Reeve JR, Bassilian S, Frost P, Hoang B, Shi Y, Lichtenstein A. (2013) Metabolomics identifies pyrimidine starvation as the mechanism of 5-aminoimidazole-4-carboxamide-1-β-riboside-induced apoptosis in multiple myeloma cells. *Mol Cancer Ther.* 12, 1310–1321.

Baruch M, Belotserkovsky I, Hertzog BB, Ravins M, Dov E, McIver KS, Le Breton YS, Zhou Y, Cheng CY, Hanski E. (2014) An extracellular bacterial pathogen modulates host metabolism to regulate its own sensing and proliferation. *Cell.* 156, 97–108.

Bassalin M, Denise H, Coombs GH, Barrett MP. (2002) Resistance to pentamidine in *Leishmania mexicana* involves exclusion of the drug from the mitochondrion. *Antimicrob Agents Chemother*. 46, 3731–3738.

Basu R, Roy S, Walden P. (2007) HLA class I-restricted T cell epitopes of the kinetoplastid membrane protein-11 presented by *Leishmania donovani*-infected human macrophages. *J Infect Dis.* 195, 1373–1380.

Ben-Sahra I, Howell JJ, Asara JM, Manning BD. (2013) Stimulation of *de novo* pyrimidine synthesis by growth signaling through mTOR and S6K1. *Science*. 339, 1323–1328.

Berman JD, Ksionski G, Chapman WL, Waits VB, Hanson WL. (1992) Activity of amphotericin B cholesterol dispersion (amphocil) in experimental visceral leishmaniasis, *Antimicrob. Agents Chemother*. 36, 1978–1980.

Berman JD, Waddell D, Hanson BD. (1985) Biochemical mechanisms of the antileishmanial activity of sodium stibogluconate. *Antimicrob. Agents Chemother*. 27, 916–920.

Bertuzzi A, D'Agnano I, Gandolfi A, Graziano A, Starace G, Ubezio P. (1990) Study of propidium iodide binding to DNA in intact cells by flow cytometry. *Cell Biophys*. 17, 257–267.

Bhowmick S, Ravindran R, Ali N. (2007) Leishmanial antigens in liposomes promote protective immunity and provide immunotherapy against visceral leishmaniasis via polarized Th1response. *Vaccine.* 25, 6544–6556.

Blanchette J, Racette N, Faure R, Siminovitch KA. Olivier M. (1999) *Leishmania*-induced increases in activation of macrophage SHP-1 tyrosine phosphatase are associated with impaired IFN-gamma-triggered JAK2 activation. *Eur J Immunol.* 29, 3737–3744.

Bogdan C, Gessner A, Solbach W, Rollinghoff M. (1996) Invasion, control and persistence of *Leishmania* parasites. *Curr. Opin. Immun.* 8, 517–525.

Boitz JM, Ullman B, Jardim A, Carter NS. (2012) Purine salvage in *Leishmania*: complex or simple by design? *Trends Parasitol.* 28, 345–352.

Boyd KE, Farnham PJ. (1997) Myc versus USF: discrimination at the cad gene is determined by core promoter elements. *Mol Cell Biol.* 17, 2529–2537.

Brooke J, Szabados E, Lyons SD, Goodridge RJ, Harsanyi MC, Poiner A, Christopherson RI. (1990) Cytotoxic effects of dihydroorotase inhibitors upon human CCRF-CEM leukemia. *Cancer Res*. 50, 7793–7798.

Callahan HL, Roberts WL, Rainey PM, Beverley SM. (1994) "The PGPA gene of *Leishmania major* mediates antimony (SbIII) resistance by decreasing influx and not by increasing efflux," *Mol Biochem Parasitol.* 68, 145–149.

Cameron MM, Acosta-Serrano A, Bern C, Boelaert M, den Boer M, Burza S, Chapman LA, Chaskopoulou A, Coleman M, Courtenay O, Croft S, Das P, Dilger E, Foster G, Garlapati R, Haines L, Harris A, Hemingway J, Hollingsworth TD, Jervis S, Medley G, Miles M, Paine M, Picado A, Poché R, Ready P, Rogers M, Rowland M, Sundar S, de Vlas SJ, Weetman D. (2016) Understanding the transmission dynamics of *Leishmania donovani* to provide robust evidence for interventions to eliminate visceral leishmaniasis in Bihar, India. *Parasit Vectors*. 27;9:25.

Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, Mottram JC, Alexander J, Plevin R. (2004) Inhibition of lipopolysaccharide-induced macrophage IL-12 production by *Leishmania mexicana* amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. *J Immunol.* 173, 3297–3304.

Carrey EA, Campbell DG, Hardie DG. (1985) Phosphorylation and activation of hamster carbamyl phosphate synthetase II by cAMP-dependent protein kinase. A novel mechanism for regulation of pyrimidine nucleotide biosynthesis. *EMBO J.* 4, 3735–3742.

Carter KC, Henriquez FL, Campbell SA, Roberts CW, Nok A, Mullen AB, McFarlane E. (2007) DNA vaccination against the parasite enzyme gamma glutamyl cysteine synthetase confers protection against *Leishmania donovani* infection. *Vaccine.* 25, 4502–4509.

Carter NS, Yates P, Arendt CS, Boitz JM, Ullman B. (2008) Purine and pyrimidine metabolism in *Leishmania*. *Adv Exp Med Biol*. 625, 141–154.

Carvalho S. Cruz T, Santarém N, Castro H, Costa V, Tomás AM. (2009) Heme as a source of iron to *Leishmania infantum* amastigotes. *Acta Trop.* 109, 131–135.

Chakraborty AK, Majumder HK. (1988) "Mode of action of pentavalent antimonials: specific inhibition of type I DNA topoisomerase of *Leishmania donovani*" *Biochemical and Biophysical Research Communications*. 152, 605–611.

Chakraborty D, Banerjee S, Sen A, Banerjee KK, Das P, Roy S. (2005) *Leishmania donovani* affects antigen presentation of macrophage by disrupting lipid rafts. *Journal of Immunology*. 175. 3214–3224.

Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M. (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol.* 5, 873–882.

Chattopadhyay A, Jafurulla M (2011). A novel mechanism for an old drug: amphotericin B in the treatment of visceral leishmaniasis. *Biochem Biophys Res Commun*. 416, 7–12.

Chen SF, Ruben RL, Dexter DL. (1986) Mechanism of action of the novel anticancer agent 6-fluoro-2-(2'-fluoro-1,1'-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt (NSC 368390):inhibition of *de novo* pyrimidine nucleotide biosynthesis. *Cancer Res.* 46, 5014–5019.

Christopherson RI, Jones ME. (1980) The overall synthesis of L-5,6-dihydroorotate by multienzymatic protein pyr1-3 from hamster cells. Kinetic studies, substrate channeling, and the effects of inhibitors. *J Biol Chem.* 255, 11381–11395.

Christopherson RI, Jones ME. (1980) The effects of pH and inhibitors upon the catalytic activity of the dihydroorotase of multienzymatic protein pyr1-3 from mouse *Ehrlich ascites* carcinoma. *J. Biol. Chem*. 255, 3358–3370.

Christopherson, R.I., Lyons, S.D., Wilson, P.K. (2006) Inhibitors of *de novo* Nucleotide biosynthesis as drugs. *Acc. Chem. Res*. 35, 961–971.

Cohen BE. (1998) Amphotericin B toxicity and lethality: a tale of two channels. *Int. J. Pharm.* 162, 95–106.

Coimbra ES, Libong D, Cojean S, Saint-Pierre-Chazalet M, Solgadi A, Le Moyec L, Duenas-Romero AM, Chaminade P, Loiseau PM. (2010) Mechanism of interaction of sitamaquine with *Leishmania donovani*. *J Antimicrob Chemother*. 65, 2548–2555.

Coleman PF, Suttle DP, Stark GR. (1977) Purification from hamster cells of the multifunctional protein that initiates *de novo* synthesis of pyrimidine nucleotides. *J. Bol. Chem*. 252, 6379–6385.

Collins KD, Stark GR. (1971) Aspartate transcarbamylase. Interaction with the transition state analogue N-(phosphonacetyl)-L-aspartate. *J. Biol. Chem.* 246, 6599–6605.

Connolly GP, Duley JA. (1999) Uridine and its nucleotides: biological actions, therapeutic potentials. *Trends Pharmacol Sci.* 20, 218–225.

Coler RN, Yasuyuki G, Bogatzki L, Raman V, Reed SG. (2007). Leish-111f,a recombinant polyprotein vaccine that protects against visceral leishmaniasis by elicitation of CD4Tcells. *Infect.Immun.* 75, 4648–4654.

Coelho AC, Beverley SM. Cotrim PC. (2003). Functional genetic identification of PRP1, an ABC transporter superfamily member conferring pentamidine resistance in *Leishmania major*. *Mol Biochem Parasitol.* 130, 83–90.

Collin, S. Davidson R, Ritmeijer K, Keus K, Melaku Y, Kipngetich S, Davies C. (2004) Conflict and kala-azar: determinants of adverse outcomes of kala-azar among patients in southern Sudan. *Clin. Infect. Dis.* 38, 612–619.

Courret N. Fréhel C, Gouhier N, Pouchelet M, Prina E, Roux P, Antoine JC. (2002) Biogenesis of *Leishmania*-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites. *J. Cell Sci*. 115, 2303–2316.

Croft SL, Engel J. (2006) Miltefosine--discovery of the antileishmanial activity of phospholipid derivatives. *Trans R Soc Trop Med Hyg*. 100 Suppl 1:S4-S8.

Cunningham AC. (2002) Parasitic adaptive mechanisms in infection by *leishmania*. *Exp Mol Pathol.* 72, 132–141.

Cunningham ML, Fairlamb AH. (1995) "Trypanothione reductase from *Leishmania donovani*—purification, characterization and inhibition by trivalent antimonials," *European Journal of Biochemistry*. 230, 460–468.

Dang Q, Song W, Xu D, Ma Y, Li F, Zeng J, Zhu G, Wang X, Chang LS, He D, Li L. (2015) Kaempferol suppresses bladder cancer tumor growth by inhibiting cell proliferation and inducing apoptosis. *Mol Carcinog.* 54, 831–840.

Dasgupta B, Roychoudhury K, Ganguly S, Kumar Sinha P, Vimal S, Das P, Roy S (2003) "Antileishmanial drugs cause up-regulation of interferon-gamma receptor 1, not only in the monocytes of visceral leishmaniasis cases but also in cultured THP1 cells," *Annals of Tropical Medicine and Parasitology*. 97, 245–257.

Das M, Kumar R, Dubey VK**.** (2015) Ornithine decarboxylase of *Leishmania donovani*: Biochemical properties and possible role of N-terminal extension. *Protein Pept Lett*. 22, 130–136.

Das S, Pandey K, Kumar A, Sardar AH, Purkait B, Kumar M, Kumar S, Ravidas VN, Roy S, Singh D, Das P. (2012) TGF-β(1) re-programs TLR4 signaling in *L. donovani* infection: enhancement of SHP-1 and ubiquitin-editing enzyme A20. *Immunol Cell Biol.* 90, 640–654.

den Boer M, Davidson RN. (2006) Treatment options for visceral leishmaniasis. *Expert Rev. Anti Infect. Ther.* 4, 187–197.

de Gontijo FA, Pascon RC, Fernandes L, Machado J Jr, Alspaugh JA, Vallim MA. (2014) The role of the *de novo* pyrimidine biosynthetic pathway in *Cryptococcus neoformans* high temperature growth and virulence. *Fungal Genet Biol.* 70, 12–23.

Denton H, McGregor JC, Coombs GH. (2004) "Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1," *Biochemical Journal*. 381, 405–412.

Descoteaux A, Turco SJ. (1999). Glycoconjugates in *Leishmania* infectivity. *Biochim. Biophys. Acta.* 1455, 341–352.

DeVito SR, Ortiz-Riaño E, Martínez-Sobrido L, Munger J. (2014) Cytomegalovirus-mediated activation of pyrimidine biosynthesis drives UDP-sugar synthesis to support viral protein glycosylation. *Proc Natl Acad Sci U S A.* 111, 18019–18024.

Dey R, Dagur PK, Selvapandiyan A, McCoy JP, Salotra P, Duncan R, Nakhasi HL. (2013) Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. *J Immunol.* 190, 2138–2149.

D'Silva C, Daunes S. (2000) Structure—activity study on the *in vitro* antiprotozoal activity of glutathione derivatives. *J. Med. Chern*. 43, 2072–2078.

Dunlop PC, Meyer GM, Ban D, Roon RJ. (1978) Characterization of two forms of asparaginase in *Saccharomyces cerevisiae*. *J Biol Chem*. 253, 1297–1304.

Dutcher JD, Gold W, Pagano JF, Vandepatte J, Amphotericin B, its production and its salts, US patent 2908,611, 1959.

Edwards BF, Fernando R, Martin PD, Grimley E, Cordes M, Vaishnav A, Brunzelle JS, Evans HG, Evans DR. (2013) The mononuclear metal center of type-I dihydroorotase from *Aquifex aeolicus*. *BMC Biochem.* 14, 14:36.

Elder RT, Xu X, Williams JW, Gong H, Finnegan A, Chong AS. (1997) The immunosuppressive metabolite of leflunomide, A771726, affects murine T cells through two biochemical mechanisms. *J. Immunol.* 159, 22–27.

Evans DR, Guy HI. (2004) Mammalian pyrimidine biosynthesis: fresh insights into an ancient pathway. *J Biol Chem*. 279, 33035–33038.

Fairbanks LD, Bofill M, Ruckemann K, Simmonds HA. (1995) Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. Disproportionate expansion of pyrimidine pools and contrasting effects of *de novo* synthesis inhibitors. *J Biol Chem*. 270, 29682–29689.

Fields C, Brichta D, Shephardson M, Farinha M, Donovan GO. (1999) Phylogenetic analysis and classification of dihydroorotase: a complex history of a complex enzyme. *Pathways Pyrimidines: Int. Newsletter*. 7, 49–63.

Fields CJ, Switzer RL. (2007) Regulation of pyr gene expression in *Mycobacterium smegmatis* by PyrR-dependent translational repression. *J Bacteriol*. 189, 6236–6245.

Forestier CL. Machu C, Loussert C, Pescher P, Späth GF. (2011) Imaging host cell–*Leishmania* interaction dynamics implicates parasite motility, lysosome recruitment, and host cell wounding in the infection process. *Cell Host Microbe*. 9, 319–330.

Forget G, Siminovitch KA, Brochu S, Rivest S, Radzioch D, Olivier M. (2001) Role of host phosphotyrosine phosphatase SHP-1 in the development of murine leishmaniasis. *Eur J Immunol*. 31, 3185–3196.

Fry M, Pudney M. (1992) Site of action of the antimalarial hydroxynaphthoquinone,2-[trans-4-(4¢-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). *Biochem. Pharmacol*. 43, 1545–1553.

Gamboa-Leon R, Paraguaide Souza E, Borja-Cabrera GP, Santos FN, Myashiro LM, Pinheiro RO, Dumonteil E, Palatnik-de-Sousa CB. (2006) Immunotherapy against visceral leishmaniasis with the nucleoside hydrolase DNA vaccine of *Leishmania donovani*. *Vaccine*. 24, 4863–4873.

Garavaglia M, Rossi E, Landini P. (2012) The pyrimidine nucleotide biosynthetic pathway modulates production of biofilm determinants in Escherichia coli. *PLoS One*. 7(2):e31252.

García-Hernández R, Gómez-Pérez V, Castanys S, Gamarro F. (2015) Fitness of *Leishmania donovani* parasites resistant to drug combinations. *PLoS Negl Trop Dis.* 7*:* e0003704. doi: 10.1371/journal.pntd.0003704.

Garg R, Gupta SK, Tripathi P, Hajela K, Sundar S, Naik S, Dube A. (2006) *Leishmania donovani*: identification of stimulatory soluble antigenic proteins using cured human, and hamster lymphocytes for their prophylactic potential against visceral leishmaniasis. *Vaccine.* 24, 2900–2909.

Getachew F, Gedamu L. (2012) *Leishmania donovani* mitochondrial iron superoxide dismutase A is released into the cytosol during miltefosine induced programmed cell death. *Mol Biochem Parasitol.* 183, 42–51.

Ghosh J, Bose M, Roy S, Bhattacharyya SN. (2013) *Leishmania donovani* targets Dicer1 to downregulate miR-122, lower serum cholesterol, and facilitate murine liver infection. *Cell Host Microbe.* 13, 277–288.

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA 3rd, Smith HO. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods.* 6:343–345.

Gibson ME. (1983) The identification of kala azar and the discovery of *leishmania donovani*. *Med Hist.* 27, 203–213.

Gierok P, Harms M, Methling K, Hochgräfe F, Lalk M. (2016) *Staphylococcus aureus* Infection Reduces Nutrition Uptake and Nucleotide Biosynthesis in a Human Airway Epithelial Cell Line. *Metabolites*. 6. pii: E41.

Gomes R, Teixeira C, Teixeira MJ, Oliveira F, Menezes MJ, Silva C, deOliveira CI, Miranda JC, Elnaiem DE, KamhawiS, Valen- zuela JG, Brodskyn CI. (2008) Immunity to a salivary protein of a sandfly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. *Proc.Natl.Acad.Sci.U.S.A.* 105, 7845–7850.

Goodwin LG (1995) "Pentostam (sodium stibogluconate); a 50-year personal reminiscence," *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 89, 339–341.

Gossage SM, Rogers ME, Bates PA. (2003) Two separate growth phases during the development of *Leishmania* in sand flies: implications for understanding the life cycle. *Int. J. Parasitol*. 33, 1027–1034.

Goto Y, Bhatia A, Raman VS, Liang H, Mohamath R, Picone AF Vidal SE, Vedvick TS, Howard RF, Reed SG. (2011) KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. *Clin Vaccine Immunol.* 18, 1118–1124.

Gourbal B, Sonuc N, Bhattacharjee H, Legare D, Sundar S, Ouellette M, Rosen BP. Mukhopadhyay R. (2004) Drug uptake and modulation of drug resistance in *Leishmania* by an aquaglyceroporin. *J Biol Chem.* 279, 31010–31017.

Gouzy A, Larrouy-Maumus G, Bottai D, Levillain F, Dumas A, Wallach JB, Caire-Brandli I, de Chastellier C, Wu TD, Poincloux R, Brosch R, Guerquin-Kern JL, Schnappinger D, Sório de Carvalho LP, Poquet Y, Neyrolles O. (2014) *Mycobacterium tuberculosis* exploits asparagine to assimilate nitrogen and resist acid stress during infection. *PLoS Pathog*. 10, e1003928.

Grande-García A, Lallous N, Díaz-Tejada C, Ramón-Maiques S. (2014) Structure, functional characterization, and evolution of the dihydroorotase domain of human CAD. *Structure*. 22,185–198.

Graves LM, Guy HI, Kozlowski P, Huang M, Lazarowski E, Pope RM, Collins MA, Dahlstrand EN, Earp HS 3rd, Evans DR. (2000) Regulation of carbamoyl phosphate synthetase by MAP kinase. *Nature*. 403, 328–332.

Greenblatt CL. (1988) Cutaneous leishmaniasis: the prospects for a killed vaccine. *Parasitol. Today*. 4, 53–54.

Grondin K, Haimeur A, Mukhopadhyay R, Rosen BP, Ouellette M. (1997) "Co-amplification of the γ-glutamylcysteine synthetase gene gsh1 and of the ABC transporter gene pgpA in arsenite-resistant *Leishmania tarentolae*," *The EMBO Journal*. 16, 3057–3065.

Guex N, Peitsch MC. (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis*. 18, 2714–2723.

Haldar AK, Sen P, Roy S. (2011) Use of antimony in the treatment of leishmaniasis: current status and future directions. *Mol Biol Int*. 571242.

Hallé M, Gomez MA, Stuible M, Shimizu H, McMaster WR, Olivier M, Tremblay ML. (2009) The *Leishmania* surface protease GP63 cleaves multiple intracellular proteins and actively participates in p38 mitogen-activated protein kinase inactivation. *J. Biol. Chem.* 284, 6893–6908.

Hamill RJ. (2013) Amphotericin B formulations: a comparative review of efficacy and toxicity. *Drugs*. 73, 919–34.

Handman E, Mitchell GF. (1985) Immunization with *Leishmania* receptor for macrophages protects mice against cutaneous leishmaniasis. *Proc.Natl.Acad.Sci. U.S.A.* 82, 5910–5914.

Hassani K, Olivier M. (2013) Immunomodulatory impact of *Leishmania*-induced macrophage exosomes: a comparative proteomic and functional analysis. *PLoS Negl Trop. Dis.* 7, e2185.

Hassani K, Shio MT, Martel C, Faubert D. Olivier M. (2014) Absence of metalloprotease GP63 alters the protein content of *Leishmania* exosomes. *PLoS ONE* 9, e95007.

Hermoso JA. (2014) Getting CAD in shape: the atomic structure of human dihydroorotase domain. *Structure*. 22, 179–81.

Hoffmann HH, Kunz A, Simon VA, Palese P, Shaw ML. (2011) Broad-spectrum antiviral that interferes with *de novo* pyrimidine biosynthesis. *Proc Natl Acad Sci U S A.* 108, 5777–5782.

Hortua Triana MA, Cajiao Herrera D, Zimmermann BH, Fox BA, Bzik DJ.(2016) Pyrimidine Pathway-Dependent and -Independent Functions of the *Toxoplasma gondii* Mitochondrial Dihydroorotate Dehydrogenase. *Infect Immun.* 84, 2974–2981.

Houslay MD, Stanley KK. (2009) Dynamics of biological membranes: influence on synthesis, structure and function, *John Wiley*, New York, p. 289.

Huang M, Graves LM. (2003) *De novo* synthesis of pyrimidine nucleotides; emerging interfaces with signal transduction pathways. *Cell Mol Life Sci.* 60, 321–336.

Ilg T, Stierhof YD, Craik D, Simpson R, Handman E, Bacic A. (1996) Purification and structural characterization of a filamentous, mucin-like proteophosphoglycan secreted by *Leishmania* parasites. *J. Biol. Chem*. 271, 21583–21596.

Irwin JJ, Shoichet BK. (2005) Zinc a free database of commercially available compounds for virtual screening. *J Chem Inf Model*. 45, 177–182.

Jackson RC, Handschumacher RE. (1970) *Escherichia coli* L-asparaginase. Catalytic activity and subunit nature. *Biochemistry*. 9, 3585–3590.

Jacobson GR, Stark GR. (1973) Aspartate transcarbamylases. In: P. D. Boyer: *The Enzymes*. 9, 22.

Jardim A, Funk V, Caprioli RM, Olafson RW. (1995) Isolation and characterization of the *Leishmania donovani* kinetoplastid membrane protein-11, a major immunoreactive membrane glycoprotein. *Biochem. J.* 305, 307–313.

Jarrod B. French, Phillip A. Yates, D. Radika Soysa, Jan M. Boitz, Nicola S. Carter, Bailey Chang, Buddy Ullman, Steven E. Ealick. (2011) The *Leishmania donovani* UMP Synthase Is Essential for Promastigote Viability and Has an Unusual Tetrameric Structure That Exhibits Substrate-controlled Oligomerization. *J Biol Chem.* 286, 20930–20941.

Jones, ME. (1980) Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. *Annu. Rev. Biochem*. 49: 253–279.

Kane MM, Mosser DM. (2000). *Leishmania* parasites and their ploys to disrupt macrophage activation. *Curr. Opin. Hematol.* 7, 26–31.

Karanja R, Ingonga J, Mwangi M, Mwala D, Lugalia R, Magambo J, Tonui W. (2011) Immunization with a combination of *Leishmania major* lipophosphoglycan(LPG) and Phlebotomus duboscqi salivary gland lysates (SGLs) abrogates protective effect of LPG against *L. major* in BALB/c mice. *Afr.J.HealthSci.* 18, 1–5.

Kaur T, Sobti RC, Kaur S. (2011) Cocktail of gp63 and Hsp70 induces protection against *Leishmania donovani* in BALB/c mice. *Parasite Immunol.* 33, 95–103.

Kaye P, Scott P. (2011) Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol*. 9, 604–615.

Khamesipour A, Dowlati Y, Asilian A, Hashemi-Fesharki R, Javadi A, Noazin S, Modabber F. (2005) Leishmanization: use of an old method for evaluation of candidate vaccines against leishmaniasis. *Vaccine.* 23, 3642–3648.

Khan S, Abdelrahim M, Samudio I, Safe S. (2003) Estrogen receptor/Sp1 complexes are required for induction of cad gene expression by 17beta-estradiol in breast cancer cells. *Endocrinology*. 144, 2325–2335.

Killick-Kendrick R. (1990) The life-cycle of *Leishmania* in the sandfly with special reference to the form infective to the vertebrate host. *Ann Parasitol Hum Comp*. 65, 37–42.

Kikuth W, Schmidt H. (1937) "Contribution to the progress of antimony therapy of kala-azar," *ChineseMedical Journal*. 52, 425–432.

Kim S, Thiessen PA, Bolton EE, Chen J, Fu G, Gindulyte A, Han L, He J, He S, Shoemaker BA, Wang J, Yu B, Zhang J, Bryant SH. (2016) PubChem Substance and Compound databases. *Nucleic Acids Res*. 44, D1202–D1213.

Kinder DH, Frank SK, Ames MM. (1990) Analogues of carbamyl aspartate as inhibitors of dihhydroorotase: Preparation of boronic acid transition state analogues and a zinc chelator carbamylhommocysteine. *J. Med. Chem.* 33, 819–823.

Krungkrai, J., Cerami, A., Henderson, G.B. (1990) Pyrimidine biosynthesis in parasitic protozoa: purification of a monofunctional dihydroorotase from *Plasmodium berghei* and *Crithidia fasciculate*. *Biochemistry*. 29, 6270–6275.

Krungkrai, S.R., Wutipraditkul, N., Krungkrai, J. (2008) Dihydroorotase of human malarial parasite *Plasmodium falciparum* differs from host enzyme. *Biochem Biophys Res Commun.* 366, 821–826.

Lane RP. (1993) Sandflies (Phlebotominae). In: Lane, R.P., Crosskey, R.W. (Eds.), *Medical Insects and Arachnids*. Chapman and Hall, London, pp. 78–119.

Laskay T, van Zandbergen G. Solbach W. (2003) Neutrophil granulocytes — Trojan horses for *Leishmania major* and other intracellular microbes? *Trends Microbiol.* 11, 210–214.

Laskowski RA, Rullmannn JA, MacArthur MW, Kaptein R, Thornton JM. (1996) AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR. *J Biomol NMR*. 8, 477–496.

Lee L, Kelly RE, Pastra-Landis SC, Evans DR. (1985) Oligomeric structure of the multifunctional protein CAD that initiates pyrimidine biosynthesis in mammalian cells. *Proc Natl Acad Sci U S A.* 82, 6802–6806.

Lee M, Chan CW, Graham SC, Christopherson RI, Guss JM, Maher MJ. (2007) Structures of ligand-free and inhibitor complexes of dihydroorotase from *Escherichia coli*: implications for loop movement in inhibitor design. *J Mol Biol.* 370, 812–825.

Lee M, Chan CW, Mitchell Guss J, Christopherson RI, Maher MJ. (2005) Dihydroorotase from *Escherichia coli*: loop movement and cooperativity between subunits. *J Mol Biol*. 348, 523–533.

Légaré D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, Haimeur A, Papadopoulou B, Ouellette M. (2001) The *Leishmania* ATP-binding cassette protein PGPA is an intracellular metalthiol transporter ATPase. *J. Biol. Chem.* 276, 26301–26307.

Leija C, Rijo-Ferreira F, Kinch LN, Grishin NV, Nischan N, Kohler JJ, Hu Z, Phillips MA. (2016) Pyrimidine Salvage Enzymes Are Essential for *De Novo* Biosynthesis of Deoxypyrimidine Nucleotides in *Trypanosoma brucei*. *PLoS Pathog.* 2016 Nov 7;12(11):e1006010.

Lemaire M, Momparler LF, Raynal NJ, Bernstein ML, Momparler RL. (2009) Inhibition of cytidine deaminase by zebularine enhances the antineoplastic action of 5-aza-2'-deoxycytidine. *Cancer Chemother Pharmacol*. 63, 411–416.

Lemke A, Kiderlen AF, Kayser O. (2005) Amphotericin B, *Appl. Microbiol. Biotechnol*. 68, 151–162.

Levine HL, Brody RS, Westheimer FH. (1980) Inhibition of orotidine-5'-phosphate decarboxylase by 1-(5'-phospho-α-d-ribofuranosyl) barbituric acid, 6-azauridine 5¢-phosphate, and uridine 5¢-phosphate. *Biochemistry*. 19, 4993–4999.

Lindsey-Boltz LA, Wauson EM, Graves LM, Sancar A. (2004) The human Rad9 checkpoint protein stimulates the carbamoyl phosphate synthetase activity of the multifunctional protein CAD. *Nucleic Acids Res.* 32, 4524–4530.

Lipscomb WN (1994) Aspartate transcarbamylase from *Escherichia coli*: activity and regulation. *Adv Enzymol Relat Areas Mol Biol*. 68, 67–151.

Li Y, Raushel FM. (2005) Inhibitors designed for the active site of dihydroorotase. *Bioorg Chem.* 33, 470–483.

Liu LX, Weller PF. (1996) Antiparasitic drugs. *N Engl J Med*. 334, 1178–1184.

Livak KJ, Schmittgen TD. (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods.* 25, 402–408.

Lodge R, Descoteaux A. (2006) Phagocytosis of *Leishmania donovani* amastigotes is Rac1 dependent and occurs in the absence of NADPH oxidase activation. *Eur J Immunol.* 36, 2735–2744.

Loh KD, Gyaneshwar P, Markenscoff Papadimitriou E, Fong R, Kim KS, Parales R, Zhou Z, Inwood W, Kustu S. (2006) A previously undescribed pathway for pyrimidine catabolism. *Proc Natl Acad Sci U S A.* 103, 5114–5119.

López-Martín C, Pérez-Victoria JM, Carvalho L, Castanys S, Gamarro F. (2008) Sitamaquine sensitivity in *Leishmania* species is not mediated by drug accumulation in acidocalcisomes. *Antimicrob. Agents Chemother*. 52, 4030–4036.

Lucas-Hourani M, Dauzonne D, Jorda P, Cousin G, Lupan A, Helynck O, Caignard G, Janvier G, André-Leroux G, Khiar S, Escriou N, Desprès P, Jacob Y, Munier-Lehmann H, Tangy F, Vidalain PO. (2013) Inhibition of pyrimidine biosynthesis pathway suppresses viral growth through innate immunity. *PLoS Pathog*.9:e1003678.

Luque-Ortega JR, Rivas L (2007) Miltefosine (hexadecylphosphocholine) inhibits cytochrome c oxidase in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother*. 51, 1327–1332.

Lusty CJ. (1978) Carbamoylphosphate synthetase I of rat-liver mitochondria. Purification, properties, and polypeptide molecular weight. *Eur J Biochem*. 85, 373–383.

Manthey MK, Huang DT, Bubb WA, Christopherson RI. (1998) Synthesis and enzymic evaluation of 4-mercapto-6-oxo-1,4-azaphosphinane-2-carboxylic acid 4-oxide as an inhibitor of mammalian dihydroorotase. *J. Med. Chem*. 41, 4550–4555.

Maran N, Gomes PS, Freire-de-Lima L, Freitas EO, Freire-de-Lima CG, Morrot A. (2016) Host resistance to visceral leishmaniasis: prevalence and prevention. *Expert Review of Anti-infective Therapy*. 14, 435–442.

Martin S, Pahari S, Sudan R. Saha B. (2010) CD40 signaling in CD8+CD40+ T cells turns on contra-T regulatory cell functions. *J. Immunol.* 184, 5510–5518.

Martins VT, Chávez-Fumagalli MA, Costa LE, Canavaci AM, Martins AM, Lage PS, Lage DP, Duarte MC, Valadares DG, Magalhães RD, Ribeiro TG, Nagem RA, Darocha WD, Régis WC, Soto M, Coelho EA, Fernandes AP, Tavares CA. (2013) Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. *PLoS Negl Trop Dis*.7:e2148.

Martiny A, Meyer-Fernandes JR, de Souza W, Vannier-Santos MA. (1999) Altered tyrosine phosphorylation of ERK1 MAP kinase and other macrophage molecules caused by *Leishmania* amastigotes. *Mol Biochem Parasitol*. 102, 1–12.

Mazumder S, Maji M, Das A, Ali N. (2011) Potency, efficacy and durability of DNA/DNA, DNA/protein and protein/protein based vaccination using gp63 against *Leishmania donovani* in BALB/cmice. *PLoSONE*. 6, e14644.

McConville MJ, Bacic A, Mitchell GF, Handman E. (1987). Lipophosphoglycan of *Leishmania major* that vaccinates against cutaneous leishmaniasis contains an alkyl glycero phosphoinositol lipid anchor. *Proc.Natl.Acad.Sci.U.S.A.* 84, 8941–8945.

McLaughlin PA, McClelland M, Yang HJ, Porwollik S, Bogomolnaya L, Chen JS, Andrews-Polymenis H, van der Velden AW. (2016) Contribution of asparagine catabolism to Salmonella virulence. *Infect Immun.* Nov 14. pii: IAI.00740-16.

Meddeb-Garnaoui A, Toumi A, Ghelis H, Mahjoub M, Louzir H, Chenik M. (2010).Cellular and humoral responses induced by *Leishmania* histone H2B and its divergent and conserved parts in cutaneous and visceral leishmaniasis patients, respectively. *Vaccine.* 28, 1881–1886.

Mehboob S, Mulhearn DC, Truong K, Johnson ME, Santarsiero BD. (2010) Structure of dihydroorotase from *Bacillus anthracis* at 2.6 A resolution. *Acta Crystallogr Sect F: Struct Biol Cryst Commun*. 66, 1432–1435.

Melby PC, Ogden GB, Flores HA, Zhao W, Geldmacher C, Biediger N M, Ahuja SK, Uranga J, Melendez M. (2000) Identification of vaccine candidates for experimental visceral leishmaniasis by immunization with sequential fractions of a cDNA expression library. *Infect. Immun.* 68, 5595–5602.

Michalska K, Jaskolski M. (2006) Structural aspects of L-asparaginases, their friends and relations. *Acta Biochim Pol.* 53, 627-640.

Mizbani A, Taheri T, Zahedifard F, Taslimi Y, Azizi H, Azadmanesh K, Papadopoulou B, Rafati S. (2009) Recombinant *Leishmania tarentolae* expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. *Vaccine.* 28, 53–62.

Mizbani A, Taslimi Y, Zahedifard F, Taheri T, Rafati S.(2011) Effect of A2 gene on infectivity of the non pathogenic parasite *Leishmania tarentolae*. *Parasitol.Res.* 109, 793–799.

Monge-Maillo B, López-Vélez R. (2016) Treatment options for visceral leishmaniasis and HIV coinfection. *AIDS Rev*. 18, 32-43.

Mookerjee Basu J, Mookerjee A, Sen P, Bhaumik S, Sen P, Banerjee S, Naskar K, Choudhuri SK, Saha B, Raha S, Roy S. (2006) "Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen activated protein kinase activation in *Leishmania donovani* infected macrophages," *Antimicrob. Agents Chemother*. 50, 1788–1797.

Moreira W, Leprohon P, Ouellette M. (2011) Tolerance to drug induced cell death favours the acquisition of multidrug resistance in *Leishmania. Cell Death Dis* 2:e201

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. (2009) Autodock4 and AutoDockTools4: automated docking with selective receptor flexiblity. *J. Computational Chemistry*. 30, 2785–2791.

Mossman T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*. 65, 55–63**.**

Mukhopadhyay R, Dey S, Xu N, Gage D, Lightbody J, Ouellette M, Rosen BP. (1996) Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. *Proc. Natl. Acad. Sci. USA*. 93, 10383–10387.

Müller K, van Zandbergen G, Hansen B, Laufs H, Jahnke N, Solbach W, Laskay T. (2001) Chemokines, natural killer cells and granulocytes in the early course of *Leishmania major* infection in mice. *Med Microbiol Immunol*. 190, 73–76.

Nadim A, Javadian E, Tahvildar- Bidruni G, Ghorbani M. (1983) Effectiveness of leishman ization in the control of cutaneous leishmaniasis. *Bull. Soc.Pathol.Exot. Filiales.* 76, 377–383.

Nakashima A, Kawanishi I, Eguchi S, Yu EH, Eguchi S, Oshiro N, Yoshino K, Kikkawa U, Yonezawa K. (2013) Association of CAD, a multifunctional protein involved in pyrimidine synthesis, with mLST8, a component of the mTOR complexes. *J Biomed Sci.* 18, 20:24.

Nandan D, Reiner NE. (1995) Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with *Leishmania donovani*: selective inhibition of signaling through Janus kinases and Stat1. *Infect Immun.* 63, 4495–4500.

Nara T, Hashimoto M, Hirawake H, Liao CW, Fukai Y, Suzuki S, Tsubouchi A, Morales J, Takamiya S, Fujimura T, Taka H, Mineki R, Fan CK, Inaoka DK, Inoue M, Tanaka A, Harada S, Kita K, Aoki T. (2012) Molecular interaction of the first 3 enzymes of the *de novo* pyrimidine biosynthetic pathway of *Trypanosoma cruzi*. *Biochem Biophys Res Commun.* 418, 140–143.

Olivier M, Gregory DJ, Forget G. (2005) Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin Microbiol Rev*. 18, 293–305.

Paila YD, Saha B, Chattopadhyay A. (2010) Amphotericin B inhibits entry of *Leishmania donovani* into primary macrophages. *Biochem Biophys Res Commun*. 399, 429–433.

Papadopoulou B, Roy G, Breton M, Kündig C, Dumas C, Fillion I, Singh AK, Olivier M, Ouel lette M. (2002) Reduced infectivity of a *Leishmania donovani* biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infect.Immun.* 70, 62–68.

Paris C, Loiseau PM, Bories C, Bréard J. (2004) Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother*. 48, 852–859.

Pathak MK, Yi T. (2001) Sodium stibogluconate is a potent inhibitor of protein tyrosine phosphatases and augments cytokine responses in hemopoietic cell lines. *J. Immunol*. 167, 3391–3397.

Peng WF, Huang CY. (2014) Allantoinase and dihydroorotase binding and inhibition by flavonols and the substrates of cyclic amidohydrolases. *Biochimie.* 101, 113–122.

Pérez-Victoria FJ, Sánchez-Cañete MP, Seifert K, Croft SL, Sundar S, Castanys S, Gamarro F. (2006) Mechanisms of experimental resistance of *Leishmania* to miltefosine: implications for clinical use. *Drug Resist. Update* 9, 26–39.

Porter TN, Li Y, Raushel FM. (2004) Mechanism of dihydroorotase reaction.*Biochemistry.* 43, 16285–16292.

Poster DS, Bruno S, Penta J, Neil GL, McGovren JP. (1981) Acivicin: An antitumor antibiotic. *Cancer Clin Trials.* 4, 327–330.

Puentes SM, Da Silva RP, Sacks DL, Hammer CH, Joiner KA. (1990) Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. *J. Immunol.* 145, 4311–4316.

Rawls JM Jr. (2006) Analysis of pyrimidine catabolism in *Drosophila melanogaster* using epistatic interactions with mutations of pyrimidine biosynthesis and beta-alanine metabolism. *Genetics*. 172, 1665–1674.

Reaves ML, Young BD, Hosios AM, Xu YF, Rabinowitz JD. (2013) Pyrimidine homeostasis is accomplished by directed overflow metabolism. *Nature*. 500, 237–241.

Rittig MG, Schröppel K, Seack KH, Sander U, N'Diaye EN, Maridonneau-Parini I, Solbach W, Bogdan C. (1998) Coiling phagocytosis of trypanosomatids and fungal cells. *Infect. Immun.* 66, 4331–4339.

Robert, X., Gouet, P. (2014) Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res*. 42, W320-W324.

Robitaille AM, Christen S, Shimobayashi M, Cornu M, Fava LL, Moes S, Prescianotto-Baschong C, Sauer U, Jenoe P, Hall MN. (2013) Quantitative phosphoproteomics reveal mTORC1 activates *de novo* pyrimidine synthesis. *Science.* 339, 1320–1323.

Robles Lopez SM, Hortua Triana MA, Zimmermann BH. (2006) Cloning and preliminary characterization of the dihydroorotase from *Toxoplasma gondii*. *Mol Biochem Parasitol.* 148, 93–98.

Rodriguez NE, Gaur U, Wilson ME. (2006) Role of caveolae in *Leishmania chagasi* phagocytosis and intracellular survival in macrophages. *Cell Microbiol*. 8, 1106–1120.

Rogers ME, Chance ML, Bates PA. (2002) The role of promastigote secretory gel in the origin and transmission of the infective stage of *Leishmania mexicana* by the sandfly *Lutzomyia longipalpis*. *Parasitology*. 124, 495–508.

Ruiz-Ramos A, Grande-García A, Ramón-Maiques S. (2015) Dihydroorotase Domain of Human CAD. *Encyclopedia of Inorganic and Bioinorganic Chemistry*.1–16. DOI: 10.1002/9781119951438.

Saudagar P, Saha P, Saikia AK, Dubey VK. (2013) Molecular mechanism underlying antileishmanial effect of oxabicyclo [3.3.1] nonanones: Inhibition of key redox enzymes of the pathogen. *Eur J Pharm Biopharm*. 85, 569–577.

Schoone GJ, Hailu A, Kroon CC, Nieuwenhuys JL, Schallig HD, Oskam L. (2001) A fast agglutination screening test (FAST) for the detection of anti-*Leishmania* antibodies. *Trans. R. Soc. Trop. Med. Hyg*. 95, 400–401.

Scianimanico S, Desrosiers M, Dermine JF, Méresse S, Descoteaux A, Desjardins M. (1999) Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmania donovani* promastigotes. *Cellular Microbiology*. 1, 19–32.

Scott HV, Gero AM, O'Sullivan WJ. (1986) In vitro inhibition of *Plasmodium falciparum* by pyrazofurin, an inhibitor of pyrimidine biosynthesis *de novo*. *Mol. Biochem. Parasitol*. 18, 3–15.

Selvapandiyan A,Dey R, Nylen S, Duncan R, Sacks D, Nakhasi HL. (2009) Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. *J. Immunol.* 183, 1813–1820.

Sharma A, Madhubala R. (2009). Ubiquitin conjugation of open reading frame F DNA vaccine leads to enhanced cell-mediated immune response and induces protection against both antimony-susceptible and –resistant strains of *Leishmania donovani*. *J. Immunol.* 183, 7719–7731.

Silva ES, Schoone GJ, Gontijo CM, Brazil RP, Pacheco RS, Schallig HD. (2005) Application of direct agglutination test (DAT) and fast agglutination screening test (FAST) for sero-diagnosis of visceral leishmaniasis in endemic area of Minas Gerais, Brazil. *Kinetoplastid Biol. Dis.* 4, 4.

Silverman JM, Chan SK, Robinson DP, Dwyer DM, Nandan D, Foster LJ, Reiner NE. (2008) Proteomic analysis of the secretome of *Leishmania donovani*. *Genome Biol.* 9, R35.

Sindermann H, Engel J. (2006) Development of miltefosine as an oral treatment for leishmaniasis. *Trans R Soc Trop Med Hyg*. 100, S17–S20.

Singh SP, Reddy DC, Rai M, Sundar S. (2006) Serious underreporting of visceral Leishmaniasis through passive case reporting in Bihar, India. *Trop Med Int Health*. 11: 899–905.

Srivastav S, Kar S, Chande AG, Mukhopadhyaya R, Das PK. (2012) *Leishmania donovani* exploits host deubiquitinating enzyme A20, a negative regulator of TLR signaling, to subvert host immune response. *Journal of Immunology*. 189, 924–934.

Stephanie St. George, Jeanette V. Bishop, Richard G. Titus, Claude P. Selitrennikoff. (2006) Novel compounds active against *Leishmania major, Antimicrob. Agents Chemother*. 50, 474–479.

Sundar S, Chakravarty J. (2008) Paromomycin in the treatment of leishmaniasis. *Expert Opin Invest Drugs*. 17, 787–794.

Suttle DP, Bugg BY, Winkler JK, Kanalas JJ. (1988) Molecular cloning and nucleotide sequence for the complete coding region of human UMP synthase. *Proc Natl Acad Sci U S A.* 85, 1754–1758.

Szempruch AJ, Dennison L, Kieft R, Harrington JM, Hajduk SL. (2016) Sending a message: extracellular vesicles of pathogenic protozoan parasites. *Nat Rev Microbiol*. 14, 669–675.

Taylor WH, Taylor ML, Balch WE, Gilchrist PS. (1976) Purification of properties of dihydroorotase, a zinc-containing metalloenzyme in *Clostridium oroticum. J. Bacteriol*. 127, 863–873.

Torrado JJ, Espada R, Ballesteros MP, Torrado-Santiago S. (2008) Ballesteros, S. Torrado-Santiago, Amphotericin B formulations and drug targeting, *J. Pharm. Sci.* 97, 2405–2425.

Tracy JW, Wbester LT. In: Hardman JG, Limbird LE, Eds. (2001) The pharmacological basis of therapeutics. New York: McGraw Hill ; pp. 1097–1120.

Truong L, Hevener KE, Rice AJ, Patel K, Johnson ME, Lee H. (2013) High-level expression, purification, and characterization of *Staphylococcus aureus* dihydroorotase (PyrC) as a cleavable His-SUMO fusion. *Protein Expr Purif*. 88, 98–106.

Turnbough CL Jr, Switzer RL. (2008) Regulation of pyrimidine biosynthetic gene expression in bacteria: repression without repressors. *Microbiol Mol Biol Rev*. 72, 266–300.

Ueno N, Wilson ME. (2012) Receptor-mediated phagocytosis of *Leishmania*: implications for intracellular survival. *Trends Parasitol.* 28, 335–344.

UN Brahmachari. (1922) "A new form of cutaneous leishmaniasis, dermal leishmanoid," *Indian Medical Gazette*. 57, 125–127.

UN Brahmachari. (1928) *A Treatise on Kala.azar*, J. Bale. Sons Danielsson, London, UK.

Underhill DM, Ozinsky A. (2002) Phagocytosis of microbes: complexity in action. *Annu Rev Immunol*. 20, 825–852.

Van Kuilenburg AB, Elzinga L, Van Gennip AH. (1998) Kinetic properties of CTP synthetase from HL-60 cells. *Adv Exp Med Biol*. 431, 255–258.

Vercesi AE, Docampo R. (1992). Ca^{2+} transport by digitonin-permeabilized *Leishmania donovani*. Effect of Ca^{2+}, pentamidine and WR-6026 on mitochondrial membrane potential in situ. *Biochemical Journal*. 284, 463–467.

Verma NK, Dey CS. (2004) Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. *Antimicrob Agents Chemother*. 48, 3010–3015.

Villela, A.D., Ducati, R.G., Rosado, L.A., Bloch, C.J., Prates, M.V., Gonçalves, D.C., Ramos, C.H., Basso, L.A., Santos, D.S. (2013) Biochemical characterization of uracil phosphoribosyltransferase from *Mycobacterium tuberculosis*. *PLoS One. 8*:e56445.doi: 10.1371/journal.pone.0056445.

Vinet AF, Fukuda M, Turco SJ, Descoteaux A. (2009) The *Leishmania donovani* lipophosphoglycan excludes the vesicular proton-ATPase from phagosomes by impairing the recruitment of synaptotagmin V. *PLoS Pathog.* 5, e1000628.

Volf P, Hostomska J, Rohousova I. (2008) Molecular crosstalks in *Leishmania*-sandfly-host relationships. *Parasite*. 15, 237–243.

Wanderley JL, Moreira ME, Benjamin A, Bonomo AC, Barcinski MA. (2006) Mimicry of apoptotic cells by exposing phosphatidylserine participates in the establishment of amastigotes of *Leishmania (L)amazonensis* in mammalian hosts. *J Immunol.* 176, 1834–1839.

Wang QY, Bushell S, Qing M, Xu HY, Bonavia A, Nunes S, Zhou J, Poh MK, Florez de Sessions P, Niyomrattanakit P, Dong H, Hoffmaster K, Goh A, Nilar S, Schul W, Jones S, Kramer L, Compton T, Shi PY.(2011) Inhibition of dengue virus through suppression of host pyrimidine biosynthesis. *J Virol*. 85, 6548–6556.

Webb B, Sali A. (2014) Comparative Protein Structure Modeling Using MODELLER. *Curr Protoc Bioinformatics*, 5.6.1–5.6.32.

Westerik J, Wolfenden R. (1974) Aspartic β semialdehyde: a potent inhibitor of *Escherichia coli* L-asparaginase. *J. Biol. Chem.* 249, 6351–6353.

World Health Organization. First WHO report on neglected tropical diseases. 2012. WHO.1-172

Available at http://whqlibdoc.who.int/publications/2010/9789241564090_eng.pdf

Williams KJ, Jenkins VA, Barton GR, Bryant WA, Krishnan N, Robertson BD. (2015) Deciphering the metabolic response of Mycobacterium tuberculosis to nitrogen stress. *Mol Microbiol.* 97, 1142–1157.

Wilson ME, Young BM, Andersen KP, Weinstock JV, Metwali A, Ali KM, Donelson JE. (1995) A recombinant *Leishmania chagasi* antigen that stimulates cellular immune responses in infected mice. *Infect Immun.* 63, 2062–2069.

Wilson ZN, Gilroy CA, Boitz JM, Ullman B, Yates PA. (2012) Genetic dissection of pyrimidine biosynthesis and salvage in *Leishmania donovani. J.Bol.Chem*. 287, 12759–12770.

Wright SD, Silverstein SC. (1983) Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J. Exp. Med.* 158, 2016–2023.

Yoshikawa K, Tanaka T, Ida Y, Furusawa C, Hirasawa T, Shimizu H. (2011) Comprehensive phenotypic analysis of single-gene deletion and overexpression strains of Saccharomyces cerevisiae. *Yeast*. 28, 349–361.

Yu CS, Chen YC, Lu CH, Hwang JK. (2006) Prediction of protein subcellular localization. Proteins 64, 643–651.

Yun MK, Nourse A, White SW, Rock CO, Heath RJ. (2007) Crystal structure and allosteric regulation of the cytoplasmic Escherichia coli L-asparaginase I. *J Mol Biol*. 369, 794–811.

Zalkin H. (1985) CTP synthetase. *Methods Enzymol*. 113, 282–287.

Zhou L, Cheng X, Connolly BA, Dickman MJ, Hurd PJ, Hornby DP. (2002) Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases. *J Mol Biol*. 321, 591–599.

Zufferey R, Mamoun CB. (2002) Choline transport in *Leishmania major* promastigotes and its inhibition by choline and phosphocholine analogs. *Mol Biochem Parasitol*. 125, 127–134.

PUBLICATIONS

- **Tiwari K**, Kumar R, Dubey VK. Biochemical characterization of dihydroorotase of *Leishmania donovani*: Understanding pyrimidine metabolism through its inhibition. *Biochimie*. 2016 131, 45-53.
- Kumar R, **Tiwari K**, Dubey VK. Methionine aminopeptidase 2 is a key regulator of apoptotic like cell death in *Leishmania donovani*. *Scientific Reports*. 2017, 7, 95 **[Research Highlights of the article published in Nature India]**
- Prabhu SV, **Tiwari K**, Suryanarayanan V, Dubey VK, Singh SK. Exploration of new and potent lead molecules against CAAX prenyl protease I of *Leishmania donovani* through Pharmacophore based virtual screening approach. *Comb Chem High Throughput Screen.* 2017, 20, 255-271
- Saikia AK, Sultana S, Devi NR, Deka MJ, **Tiwari K**, Dubey VK. Diastereoselective synthesis of substituted hexahydrobenzo[de]isochromanes and evaluation of their antileishmanial activity. *Org Biomol Chem*.; 2016, 14, 970-979.

CONFERENCE PROCEEDINGS AND WORKSHOPS

- **Kartikeya Tiwari**, Vikash Kumar Dubey*. Role of aspartate metabolism in the growth and infectivity of *Leishmania donovani*. 3rd International conference on PCSMM held at Bose Institute, Kolkata, West Bengal, India, January 08-10, 2017.
- **Kartikeya Tiwari**, Vikash Kumar Dubey*. Unravelling the role of L-asparaginase in the growth and infectivity of *Leishmania donovani*. 57th annual conference of Association of Microbiologists of India (AMI) held at Gauhati University, Guwahati, Assam, India, November 24-27, 2016.
- **Kartikeya Tiwari**, Vikash Kumar Dubey*. Investigating pyrimidine metabolism of *Leishmania donovani* through dihydroorotase mediated inhibition. 9th annual TCS event and Flow cytometry workshop (FABACTCS 2016) held at IIT Guwahati, Guwahati, Assam, India. November 03-05, 2016.
- **Kartikeya Tiwari**, Vikash Kumar Dubey*. Understanding pyrimidine metabolism in *Leishmania donovani* physiology through dihydroorotase mediated inhibition. Research conclave'16 held at IIT Guwahati. Assam, India. March 17-20, 2016.
- **Kartikeya Tiwari**, Vikash Kumar Dubey*. Exploring *Leishmania donovani* dihydroorotase as an effective drug target for antileishmanial therapy. Recent Developments In Medical Biotechnology And Structure Based Drug Designing (RDMBSBDD) held at IIT Guwahati. Assam, India, December 06-07, 2015.
- Mousumi Das, **Kartikeya Tiwari**, Shalini Singh, Ruchika Bhardwaj, Ritesh Kumar, Gundappa Saha, Adarsh Kumar Chiranjivi, Kamalesh Verma, Sudipta Bhattacharya, Bijoy Mukut Borgohain and Prof. Vikash Kumar Dubey* . Hacking the biology of *Leishmania* parasite. Research Conclave held at IIT Guwahati. Assam, India. March 2015.