Studies on redox system of *Leishmania donovani*: Understanding drug resistance process and discovery of novel drug candidates

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

DOCTOR OF PHILOSOPHY

By

Ms. Mousumi Das

Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
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MAY 2015

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DEDICATED

TO

PARENTS

TEACHERS

AND ALMIGHTY GOD

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

STATEMENT

I hereby declare that the matter embodied in this thesis entitled "**Studies on redox system of *Leishmania donovani*: Understanding drug resistance process and discovery of novel drug candidates**" 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.

Date: MAY 2015

Mousumi Das

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

CERTIFICATE

It is certified that the work described in this thesis entitled "**Studies on redox system of *Leishmania donovani*: Understanding drug resistance process and discovery of novel drug candidates**" by **Ms. Mousumi Das (**Roll No: 10610621), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of her 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 any kind of degree.

Prof. Vikash Kumar Dubey
(Thesis Supervisor)

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Mousumi Das
May 2015
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ABBREVIATIONS

BMJ	:	British Medical Journal
WHO	:	World health organization
HIV	:	Human immunodeficiency virus
DDT	:	Dichlorodiphenyltrichloroethane
CL	:	Cutaneous leishmaniasis
ML	:	Mucocutaneous leishmaniasis
VL	:	Visceral leishmaniasis
PKDL	:	Post kala-azar-dermal leishmaniasis
NVBDCP	:	National Vector Borne Disease Control Programme
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M
IFA	:	Indirect immunofluorescent-antibody
DAT	:	Direct agglutination
DNA	:	Deoxyribonucleic acid
PCR	:	Polymerase chain reaction
ELISA	:	Enzyme-linked immunosorbent assay
IFN	:	Interferon
TryS	:	Trypanothione Synthetase
$T(SH)_2$	:	Trypanothione
TryR	:	Trypanothione reductase
GR	:	Glutathione reductase
H_2O_2	:	Hydrogen peroxidise
ODC	:	Ornithine decarboxylase
DMFO	:	α-Difluoromethylornithine
APA	:	3-aminooxy-1-aminopropane
T-*Ld*ODC	:	Truncated-*Leishmanial* ODC

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Chapter I

Review of literature on *Leishmania* redox metabolism, drug resistance and drug discovery.*

1.1 Abstract

Leishmaniasis is a disease caused by a human parasite *Leishmania*, spread by the bite of a blood feeding infected sandfly. There are very few drugs available against the disease with severe side effects. Leishmaniasis has three different forms (Cutaneous, Mucocutaneous and Visceral). Most deadly form of Leishmaniasis is visceral leishmaniasis. If the patient is left untreated, it may lead to death. Proper drug treatment can cure the disease but drug resistance developed by the parasite against most of the existing drugs is a major challenge. Therefore, there is a need for novel and better drugs. Moreover, it is also necessary to understand drug resistance mechanism in order to conquer this deadly disease. Some enzymes and pathways which play crucial role for parasite survival can be targeted for the development of new drugs. Combinatorial therapy may be a better option, which has lower side effects and less occurrence of drug resistance problem.

Keywords: Leishmaniasis; Drugs; Side effects; Resistance; Drug targets

*****Part of the review is communicated for publication

1.2 Introduction

Leishmaniasis is a very old disease; older than the date reported. It is found to be endemic in many parts of the world. Initially, leishmaniasis was confused with malaria. But later on in early 1900, Sir William Boog Leishman reported presence of peculiar bodies in spleen of soldier suffering from fever at Netely hospital. He published this finding in British Medical Journal with a title "On possibility of the occurrence of trypanosomiasis in India" in 1903. Charles Donovan, a professor of Madras Medical College, reported the presence of similar peculiar bodies in spleen of a patient suffering from fever with splenomegaly. He also reported this finding same year (1903) and concluded that this organism is new divergence of *Trypanosome*. The parasite was named as *Leishmania donovani* in honour of Sir William Boog Leishman and Charles Donovan, who discovered the parasite in human blood and subsequently did pioneering research on the parasite (Figure 1.1).

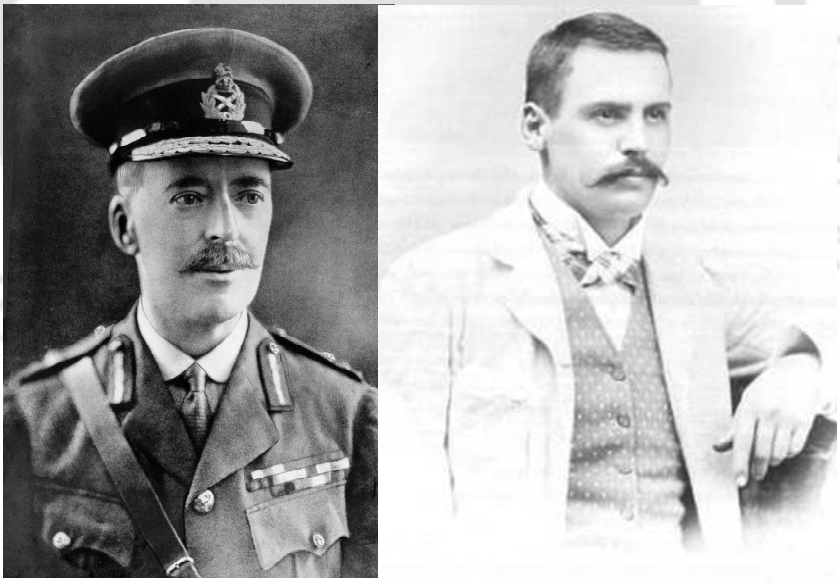


Figure 1.1: Sir William Boog Leishman and Charles Donovan, the pioneers of leishmaniasis, who discovered for the first time the parasite in human blood, and in the honour of the name of the two people the parasite named as *Leishmania donovani*. [***Source of Images***: Wikipedia]

Leishmaniasis is known by different names at different places. In Assam, people started suffering from this disease in the year 1882 and McNaught, a Civil Medical Officer of Tura, headquarter of Garo hills district that time in Assam (now in Meghalaya), named it as "Kala-azar". The parasite completes its life cycle in two different hosts (human and sandfly). The disease spreads by the bite of blood feeding infected sand flies (dipterans) of genera: *Phlebotomus* (old world) and *Lutzomyia* (new world), subfamily: *Phlebotominae,* family: *Psychodidae* (*Stuart et al., 2008; Schmidt et al., 2012*). There are 500 known species of sandfly, of which only thirty are found to transmit leishmaniasis. Sandfly is of 2-3 mm size. Only female sandfly feed on blood meal for getting proteins from human blood to lay eggs (WHO). *Leishmania* lives inside the gut of sand flies by incorporating some adaptations, which includes secretion of phosphoglycans (helps in protecting parasite from insect digestive enzymes), producing chitinases (which degrades sand fly's stomodeal valve), neuropeptide secretion (seize midgut-hindgut peristalsis), and attachment to the midgut to avoid elimination (*Kamhawi, 2006*). There are over thirty different species of *Leishmania* around the world belonging to family *Trypanosomatidae,* out of which, approximately twenty are reported to be pathogenic for mammals (Table 1.1). However, *Leishmania donovani* is the most common species found in India.

Table 1.1: Taxonomic classification of *Leishmania donovani* (data taken from https://web.stanford.edu/class/humbio103/ParaSites2003/Leishmania/Class%20and%20Morph.html)

Domain	Classification
Kingdom	Protista (*Haeckel, 1866*)
Sub-kingdom	Protozoa (*Goldfuss, 1817*)
Phylum	Sarcomastigophora (*Honigberg and Balamuth, 1963*)
Sub-phylum	Mastigophora (*Deising, 1866*)
Class	Zoomastigophora (*Calkins, 1909*)
Order	Kinetoplastida (*Honigberg, 1963*)
Family	Trypanosomatidae (*Doflein, 1901*)
Genus	*Leishmania* (*Ross, 1903*)
Species	*donovani* (*Laveran et Mesnil, 1903*)

1.2.1 Life cycle of *Leishmania:* Life cycle stage of *Leishmania* is shown in Figure 1.2. When an infected sandfly feeds on human blood, it injects *Leishmania* promastigote (Figure 1.2A). In human blood, promastigote is exposed to many host defence cells like neutrophils, macrophage and dendritic cells. Macrophages phagocytose promastigotes into a parasitophorous vacuole, which fuses with lysosomes to generate an acidic compartment containing hydrolytic enzymes (*Lynn et. al., 2011*). Inside the macrophages, it further differentiates into amastigote (Figure 1.2C) and undergoes multiplication by binary fission. Macrophage bursts and amastigotes get scattered inside the body and infect nearby uninfected macrophages. Table 1.2 shows differences between various life cycle stages. Amastigotes infects different parts of the body depending upon the type of species involved. Infected organisms become reservoir of the pathogen. When another sandfly feeds on blood containing infected macrophage, it ingests macrophage containing amastigote. Inside the gut of sandfly, amastigote converts into promastigotes which moves to proboscis and get ready for another set of infection. This phase of *Leishmania* life cycle is called anthroponotic, where it involves only human beings. But when sandfly carries the parasite from human to any other animal, this stage of life cycle is called zoonotic where the animal plays a role of reservoir. Examples of animals are pig, cats, donkeys, mule, dog, horses and squirrels etc. Schematic representation of *Leishmania* life cycle is shown in Figure 1.3.

Change in pH and temperature leads to transition of parasite between promastigote and amastigote in sandfly and human respectively, but occurrence of both stages of parasite together was reported in a patient suffering from cutaneous leishmaniasis (*Haouas et al., 2014*). In the cutaneous form, parasite which is present in outer lesion may have been exposed to different temperature and hence gets converted into another form (Figure 1.4). Conversion of non-infective procyclic stage to highly infective metacyclic stage is called metacyclogenesis, which occurs in the gut of sandfly and the genes involved in metacyclogenesis are Mat-1-a, SHERP and HASP (*Muskus et al., 2002*; *Silva et al., 1987*). Some of the reports has stated that stress conditions may lead to metacyclogenesis, but some other report has postulated that metacyclogenesis is inhibited by addition of adenosine (*Serafim et al., 2012*).

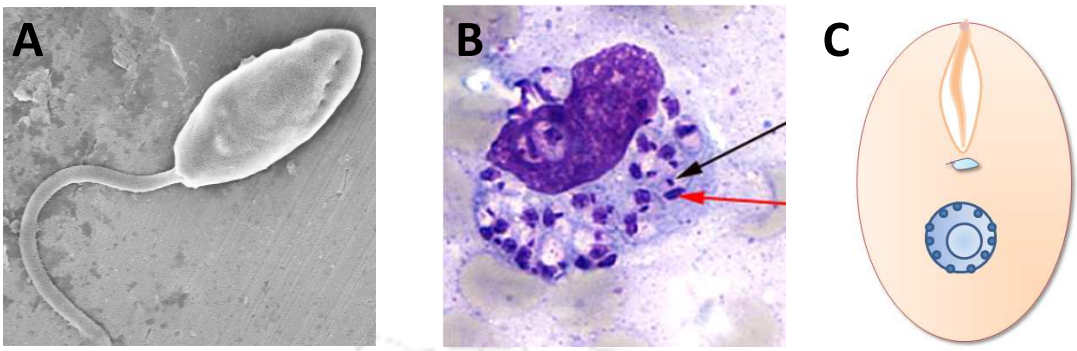


Figure 1.2: Different life cycle stages of *Leishmania* **(A)** Promastigote stage of *Leishmania*, **(B)** Amastigote stage of *Leishmania* inside infected macrophage, stained by geimsa. ***Source***: http://www.cdc.gov/parasites/leishmaniasis/health_professionals/ **(C)** Amastigote stage of *Leishmania* without flagella and round body

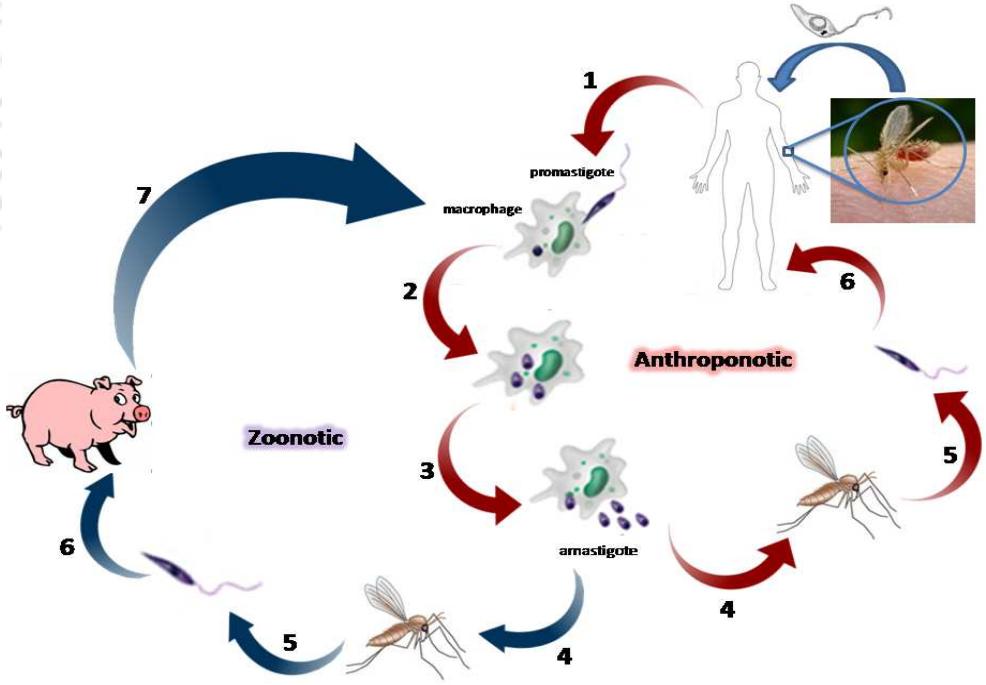


Figure 1.3: Life cycle of *Leishmania* parasite and its mode of transmission: the parasite has two different hosts, human and sandfly. If animal act as a reservoir than it is called as Zoonotic (animal to vector to human) and if human is involved than it is called anthroponotic (human-vector-human) which is common in India.

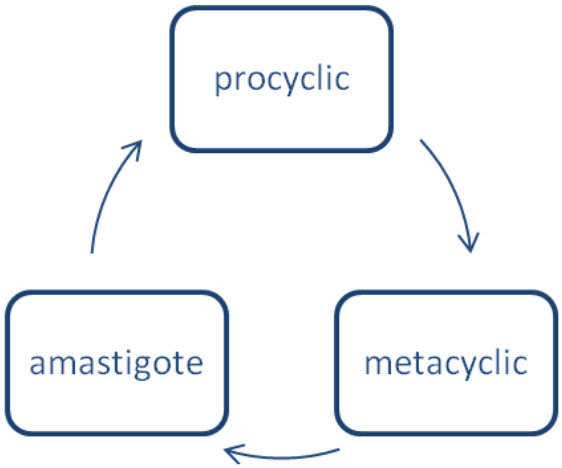


Figure 1.4: Metacyclogenesis: The parasite passes through different stages: procyclic stage where it is multiplicative but poorly infective, then it becomes metacyclic inside insect gut where it becomes highly infective but non dividing promastigote and inside macrophage it transforms into amastigote stage.

Table 1.2: Differences between amastigote and promastigotes stages of *Leishmania*: In laboratory, promastigotes can be converted into amastigote by changing pH of media 5.5-6.0 and at 37°C in CO_2 incubator in 120 hours.

Sl. no.	Promastigote	Amastigote	Reference
1	With motile flagella	Rudimentary non-motile flagella	*Gluenz et al.,2010*
2	Extracellular	Intracellular	*Sadick et al.,1985*
3	Thin & elongated, 5-14 µM in length,1.5-3.5 µM in width	Small & spherical, 2-4 µM diameter	*Royer et al., 2002*
4	Neutral-alkaline pH	Acidic pH	*Glaser et al.,1988*
5	25°C temperature	37°C temperature	*Zilberstein et al., 1994*
6	Proline as carbon source	Sugar as carbon source	*Mazareb et al., 1999*

It is suggested that the genus *Leishmania* might have originated in South America and later diversified after migration into Asian countries like Bangladesh, India, Nepal, Bhutan etc (*Lukes et al., 2007*). According to WHO, 14 million people from 88 countries, mainly from five continents i.e., Asia, Europe, Africa, South America and North America are suffering from Leishmaniasis. Moreover, 350 million people are at risks of infection and around 1.5 to 2 million new cases are arriving annually. The disease existed for many years in Bengal and China and was first recognized in 1824 in Jessore, India. According to a report, research on Leishmaniasis includes total 184 trials with sample size of 23,039 (*Kappagoda et al., 2012*). It is also the second most neglected parasitic disease next to malaria (*Singh et al., 2012*). Epidemiological survey (*Khan et al., 2013)* on visceral leishmaniasis in India is shown in Figure 1.5B. It was reported that 75% of cases are male and only 25% of cases are female (*Khan et al., 2013*). This disease is mostly prevalent in male may be because the sufferers are mainly poor people where male go out for work and hence more exposed to vector. The age wise distribution of the disease indicates that 75% people are from the age group of between 15 to 45 years (Figure 1.5A). This may be because of the fact that people of this age group are more active as they go out for work and get exposed to vector making them more susceptible to leishmaniasis (*Khan et al., 2013).*

World health organization (WHO) and Government of India have decided to eradicate the disease by year 2010, but new cases are still emerging from several parts of world. Now this disease is not only prevalent in endemic region but also spreading to non-endemic region because of migration of people. For example: There was a recent outbreak in Gujarat: moreover, sporadic cases have also been reported in 2007 (*Sharma et al., 2007*). However, the disease is spreading to non-endemic areas as well. Recently, for the first time total twenty cases of visceral leishmaniasis were reported from Doda district of Jammu and Kashmir. Since March 2013, 750 people have died because of the disease in the past five years. Few cases emerged from Kerala and Madhya Pradesh also. The reason of fatality may be due to lack of proper vector control program and re-emergence of visceral leishmaniasis due to HIV-coinfection (*Muniaraj, 2014*).

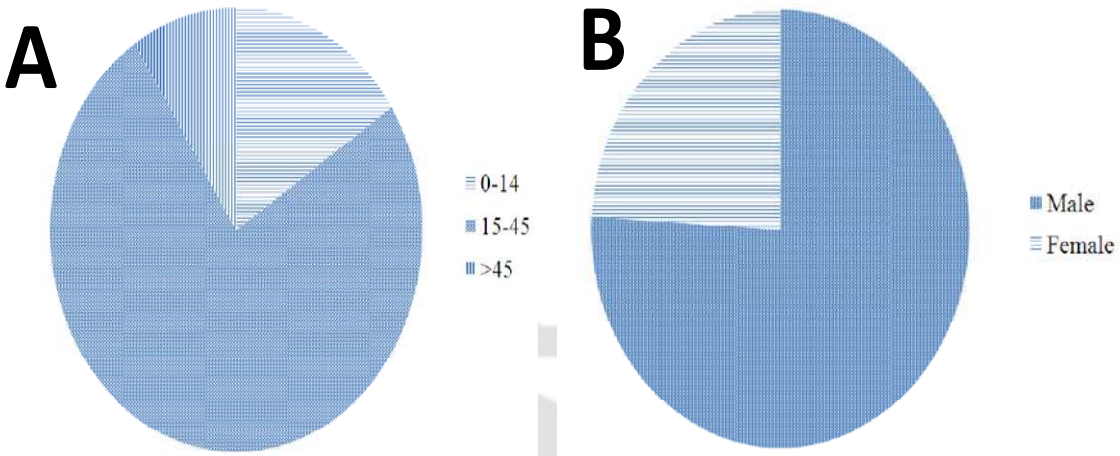


Figure 1.5: Pie chart mode of representation showing: (A) Distribution of visceral leishmaniasis cases based on age, people from age group of 15-45 are more infected by the parasite; (B) Distribution of visceral leishmaniasis cases based on sex, male get more affected by the disease (75%) (***Source:*** *Am J Infect Dis*. 9, 59−62, 2013).

Vector born disease like Lieshmaniasis cannot be controlled only by using drugs. One of the most important disease control strategies is vector control programme. An effective and well planned vector control program would be beneficial to control the spread of disease. In India, vector control programme was initiated long back since 1977. Vector control programme using Dichlorodiphenyltrichloroethane (DDT), a well known insecticide, was initiated three times since 1977 to 2007 (Figure 1.6). As shown in the Figure 1.6, during the time of vector control programme, number of cases and deaths were drastically lower whenever DDT spray was done (*Muniaraj, 2014*). As the effectiveness of DDT fades with time, increases in number of cases are seen. The data available in the literature (*Muniaraj, 2014*) clearly hints towards usefulness of continuous and proper vector control program because the parasite cannot complete its life cycle without sandfly. Instead of DDT, other insect repellent can also be used, for example pyrethenoides are used in Bangladesh and Nepal instead of DDT. It is worth mentioning that the environmental impact of the vector control programme and effect of insecticides on environment may also be taken into consideration.

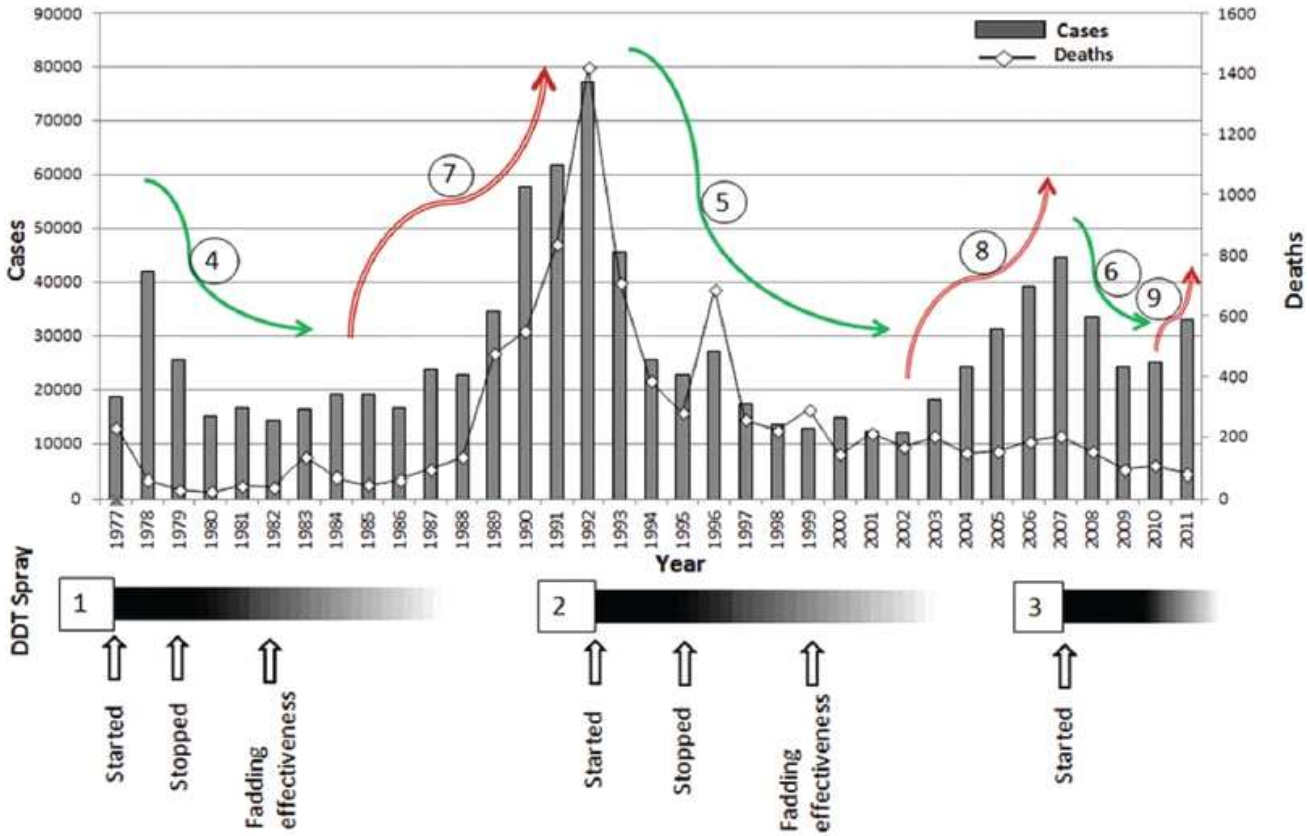


Figure 1.6: Number of cases and deaths compared with DDT spray (vector control programme): DDT spray was done three times from 1977 to 2007 in India, during those days number of cases decreased but with the fading efficacy of DDT again the numbers have increased (***Source***: figure adopted from *Tropical Parasitology, 2014*, 4, 10–19).

1.2.2 Geographical distribution of leishmaniasis: The disease is endemic in many part of globe, for example, India, Bangladesh, Pakistan, China, Africa, Southern Europe, South America and Russia (Figure 1.7). In India, 90% of the cases are from northern parts of the country, which includes Bihar, Uttar Pradesh, West Bengal etc, where Bihar is the most severely affected region. Leishmaniasis is also found in Orissa, and Madras which are hot and humid places and hence favourable for vector growth and survival. Total cases and deaths due to *Leishmaniasis* in India have shown in the form of pie chart in Figure 1.8.

Depending upon the species involved, there are three clinical forms of the disease as shown in Figure 1.9: cutaneous, mucocutaneous and visceral.

Cutaneous leishmaniasis: as the name suggest the parasite affects the skin. It is the most common form of the disease across the globe. The disease is not very dangerous and can be cured but if left untreated it may lead to other dangerous form of the disease. Cutaneous leishmaniasis is prevalent in Saudi Arabia, Afghanistan, Peru, Brazil and Iran and is caused by *L. tropica, L. aethiopica, L. major, L. mexicana, L. guyanensis, L. amazonensis* and *L. braziliensis*.

Mucocutaneous leishmaniais: this form of the disease affecting mainly the mouth and nasal part (mucosal) of body and is prevalent in South America, especially in Brazil, Paraguay, Ecuador, Bolivia, Peru, Colombia, and Venezuela. It is caused by *L. panamensis* and *L. braziliensis*.

Visceral leishmaniasis: this form is common in India, 90% of cases are from northern part of India and is caused by *L. donovani, L. chagasi* and *L. infantum* species (*Muniaraj, 2014*). It is also prevalent in Bangladesh, Sudan, and Brazil. This is the most dangerous form of the disease as it mainly affects internal vital organs of the body (spleen, bone marrow, liver), hence fatal. Its symptoms include darkening of the skin after infection, and hence it is also called as black fever or kala-azar. PKDL: Post kala-azar-dermal leishmaniasis occurs after recovery from leishmaniasis. It is difficult to detect and needs prolong-expensive treatment.

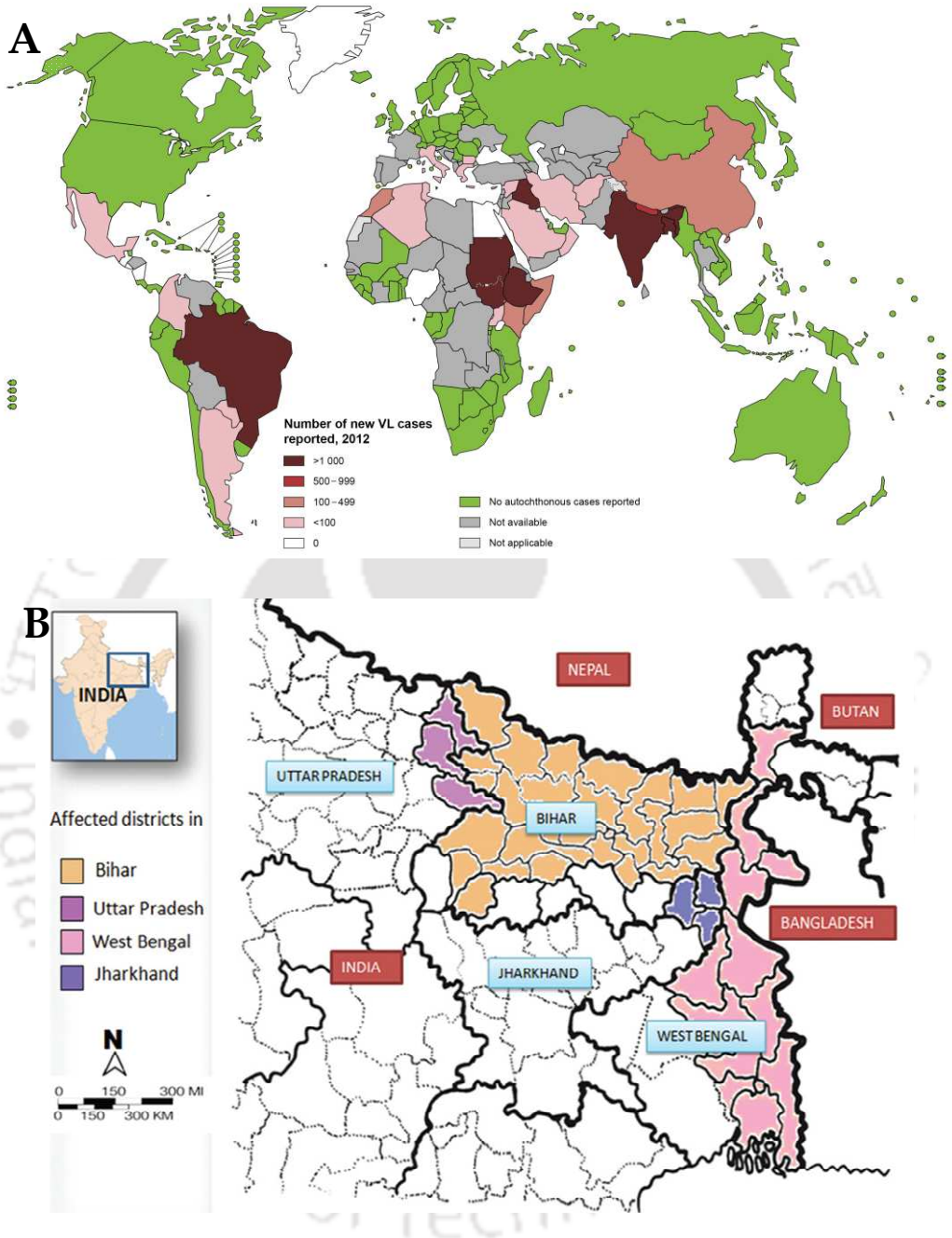


Figure 1.7: Geographical distribution of *Leishmaniasis*: (A) Around world: it is endemic in many places in India, China, Africa, Southern Europe, South America and Russia; Source: http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_VL_2013.png?ua=1 **(B)** In India: it is especially common in Assam and Bengal along the coasts of the Ganges and the Brahmaputra. It is also endemic in Bihar, Orissa, Madras and eastern parts of Uttar Pradesh, which are hot and humid places favourable for vector growth. (Figure adopted from *Tropical Parasitology, 2014,* 4, 10–19).

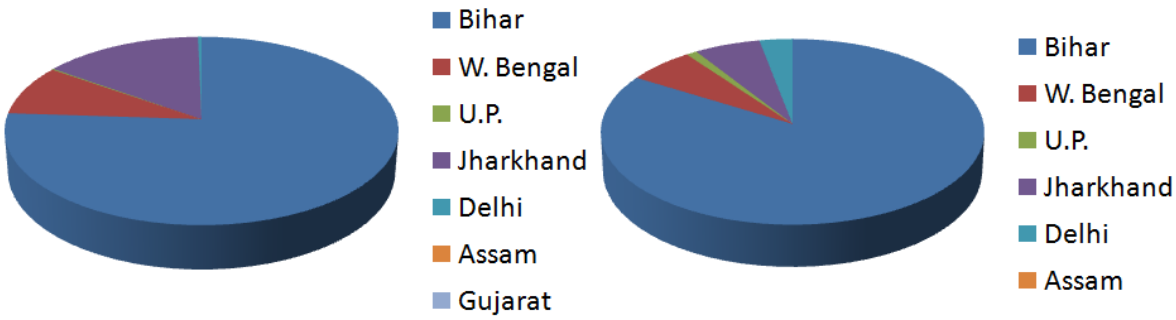


Figure 1.8: Distribution of *Leishmania* cases in India: (A) Percentage of visceral leishmaniasis cases in India, **(B)** Percentage of deaths (data taken from *National Vector Borne Disease Control Programme, 2014* is re-plotted in Pie chart)

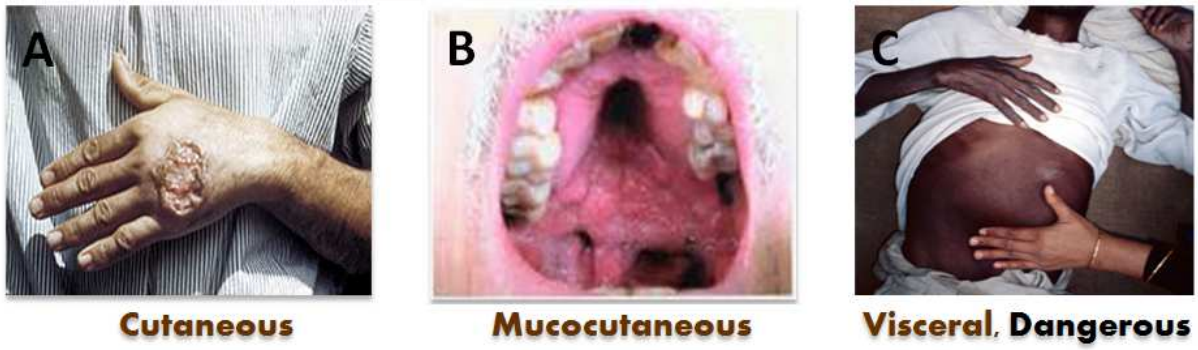


Figure 1.9: Different forms of leishmaniasis: (**A)** Cutaneous leishmaniasis: affects skin; **(B)** Mucocutaneous leishmaniasis: affects mouth and nose; **(C)** Visceral leishmaniasis: affects internal organs. (***Source:*** Wikipedia or other web resources)

1.2.3 Symptoms of visceral Leishmaniasis: Visceral Leishmaniasis, commonly known as Kala-azar in India is most deadly form of the disease. Symptoms of visceral leishmaniasis includes long term fever, weight loss, anaemia or low hemoglobin level (*Sharma et al., 2007*), hepatosplenomegaly, pancytopenia (*Lindoso et al., 2012; Burza et. al., 2014*), hyper-gammaglobulinemia (*Lindoso et al., 2012*), loss of appetite, dry-thin-scaly skin, hair loss and darkening of skin.

1.2.4 Detection of Leishmaniasis: Sometimes the diagnosis of leishmaniasis is difficult, but peripheral blood smears of buffycoat and blood culture may give correct detection (*Vector Borne Disease Control Programme, 2014*). Detection of this disease is problematic because the symptoms are very much similar to those of malaria, tuberculosis and typhoid. Elevated levels of the two antibodies, IgG and IgM have been observed during infection. Indirect immunofluorescent-antibody test (IFA) and direct agglutination test (DAT) are most common techniques used for measuring antibody levels (*Mikaeili et al., 2007*). DAT is found to be more sensitive and specific than IFA test. Following are different methods by which occurrence of leishmaniasis in a patient can be detected:

i. Examination of slides (biopsy specimens, impression smears, dermal scrapings)
ii. Splenic aspirate examination by Giemsa staining
iii. Detection of kinetoplast DNA by PCR
iv. Real time quantification of enzymes present in spleen, liver and bone marrow tissue.
v. ELISA (enzyme-linked immunosorbent assay) measure antibody profile or rK39 Rapid Test
vi. Measurement of cytokines in body (IFN-γ, IL-4, IL-10)
vii. Estimation of cell population in the blood (T CD4+/CD8+; T CD4+CD25+; B cells) (*Practical Guide for Specimen Collection and Reference Diagnosis of Leishmaniasis, 2014*).
viii. A complete blood count, liver enzyme analysis and renal profile testing of patient for 4 weeks (*Sousa et al., 2011*).

1.2.5 Redox Metabolism in *Leishmania:* There are several differences in biochemical and metabolic pathways of host and parasite. These differences can be exploited to discover drug targets. *Leishmania* and other trypanosomes have a unique thiol based redox metabolism pathway involving trypanothione, $T(SH)_2$ as shown in Figure 1.10. Trypanothione is produced by Trypanothione synthetase (TryS), which provides reducing equivalents (anti-oxidant property) to different proteins within cells which in turns saves the parasite from oxidative damage (scavenge free radicals like H_2O_2, $ONOO^-$, NO etc) and is also involved in the formation of deoxyribonucleotides (*Krauth-Siegel et al., 2008*). Thiol based redox

metabolism is not present in human counterparts and is replaced by glutathione and both have different substrate specificity. $T(SH)_2$ is maintained in reduced state by trypanothione reductase (TryR). The enzyme has different substrate specificity compared to glutathione reductase (GR). So it is a good drug targeting enzyme. Few known inhibitors of TryR are phenothiazine and related tricyclic compounds. Trypanothione reductase (TryR) is the most studied enzyme of thiol based system for discovering inhibitors, but inhibition has to be reached by 90% in order to affect viability of the parasite. Instead TryR, TryS is found to be most promising (*Irigoin et al., 2008*). *Leishmania* does not have catalase enzyme. Removal of hydrogen peroxidase (H_2O_2) is done by $T(SH)_2$, tryparedoxin (TXN) and peroxiredoxin (PRX). *Leishmania* also expresses unusual plant-like ascorbate peroxidases. Several reports suggested that *Leishmania* eEF1B may contribute to the protection against lipid peroxidation in the parasite.

Polyamine biosynthesis pathway is another pathway present in parasite which helps in the redox metabolism. Ornithine decarboxylase (ODC) is the initial and rate limiting enzyme in polyamine biosynthesis which converts ornithine to putrescine. Putrescine in turn gets converted to higher polyamines that are spermidine and spermine. Spermidine is the precursor of trypanothione. Spermidine combines with two molecules of glutathione to form trypanothione (*Fairlamb et al., 1992*). Therefore, formation of spermidine is very necessary to regulate thiol redox metabolism pathway. Polyamines play critical role in structure and function of DNA, increase rigidity of cell membrane by interacting with phospholipids and proteins in membrane (*Wallace et al., 2003*). Thus, the polyamine synthesis pathway ultimately helps in growth and survival of the parasite (*Heby et al., 2003*). Disruption of this pathway would make the parasite susceptible to oxidative stress and also disable their ability to replicate. An ornithine decarboxylase knockout (Δodc) strain lacking both ODC alleles was incapable of growing in polyamine-deficient medium (*Jiang et al., 1999*). This auxotrophy could be overcome by addition of putrescine or spermidine and not spermine in the media. *Leishmania donovani* ornithine decarboxylase (*Ld*ODC) is indispensable for parasite survival in the mammalian host (*Boitz et al., 2009*). Studies demonstrate that *L. donovani* amastigotes require ODC activity to sustain a robust infection in mice and are not capable of scavenging polyamines from the host milieu in amounts sufficient to sustain an

infection. These findings indicate that targeting ODC is a rational therapeutic paradigm for the treatment of leishmaniasis. *Leishmania donovani* ornithine decarboxylase can be a potential target that would inhibit formation of spermidine and subsequently trypanothione, thereby affecting the growth and survival of *Leishmania.*

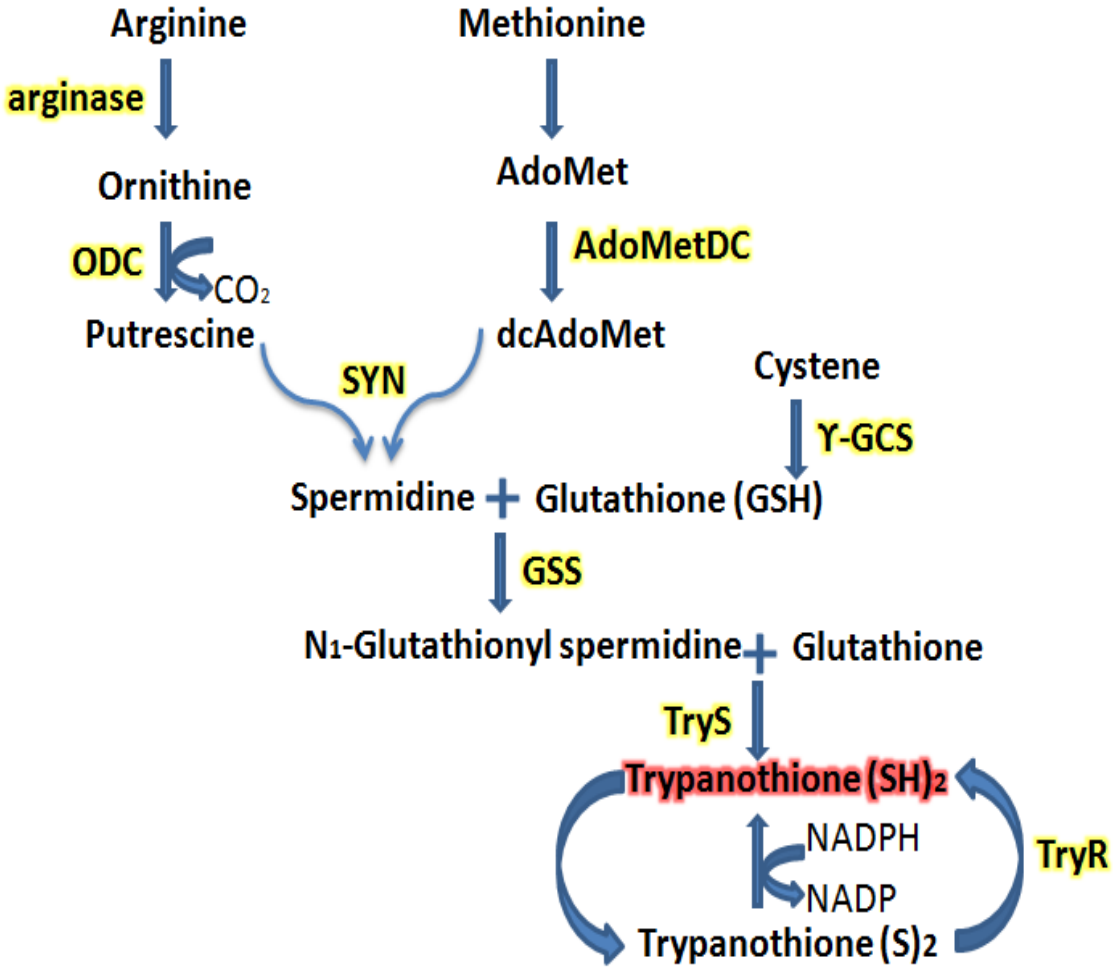


Figure 1.10: Enzymes involved in thiol based redox metabolism of *Leishmania*: Ornithine decarboxylase (ODC) is rate limiting enzyme, which converts ornithine to putrescine, which is further converted to spermidine by spermidine synthase (SYN). Spermidine combines with two molecules of glutathione to forms trypanothione by trypanothione Synthetase (TryS), which further helps in scavenging reactive oxygen species (ROS). Trypanothione is maintained in reduced state by trypanothione reductase (TryR).

1.2.6 Ornithine decarboxylase of *Leishmania:* Ornithine decarboxylase is the first rate limiting enzyme of redox metabolism involving trypanothione and polyamine biosynthetic pathway in *Leishmania*. Inhibiting *Ld*ODC may inhibit formation of trypanothione, thereby affecting the growth and survival of the parasite. Ornithine decarboxylase is also present in human counterpart showing 39% amino acid sequence similarity with *Ld*ODC. The enzyme, *Ld*ODC, appears to have a half-life in excess of 6 hours compared to human ODC and there is no detectable intracellular turnover (*Mukhopadhyay et al., 1995*). Because of these differences, *Ld*ODC can be exploited for therapeutic agents. Polyamine biosynthetic pathway helps in stabilizing DNA and inhibits endonuclease, important for cell growth and differentiation. Ornithine decarboxylase of *L. donovani* (EC 4.1.1.17) was cloned already in 1992 (*Hanson et al., 1992*). Crystal structure of Human ODC was resolved which opened-up more structural insights (*Almrud et al., 2000*). According to one report trypanosome ornithine decarboxylase is stable because it lacks strong PEST sequences found in the carboxyl terminus of the mouse enzyme which targets intracellular degradation (*Ghoda et al., 1990*). One published work clearly confirms that *Ld*ODC has kinetic properties that are distinct from those of the human ODC (*Dufe et al., 2007*). A potent inhibitor of *Ld*ODC, 3-aminooxy-1-aminopropane (APA), isosteric analogue of putrescine has *Ki* value of 1 nM, which actually was somewhat lower than that found for the human ODC enzyme. D,L-α-difluoromethylornithine (DMFO) is a specific and irreversible inhibitor of ODC (*Dufe et al., 2007*). DFMO, is also known as Eflornithine (Table 1.3). Side effect of DFMO is hearing loss in human (*Doyle, 2001*). DFMO is very specific inhibitor of ODC and may be due to this reason, parasite has gained resistance, and *L. donovani* is found to be resistant towards DFMO. Like all drugs have side effects, DFMO also have some side effects like: seizures, fever, infections, neutropenia, hypertension, diarrhea and septic shock occurs (*Lee et al., 2005*). One report demonstrated that *Entamoeba histolytica* ODC (*Eh*ODC) is functional in dimeric form (*Preeti et al., 2012*). They made 3D model of dimeric *Eh*ODC which opens up possibilities for alternate enzyme inhibition strategies by targeting the dimer disruption. *Ld*ODC is a cytosolic soluble enzyme and continuously expressed by both promastigotes and amastigotes, so ODC activity assay can be used for quantification of cell number (*Ephros et al., 1997*).

Table 1.3: Inhibitors of ornithine decarboxylase, mode of inhibition and their IC_{50} value comparison on promastigote and amastigote

Sl.no.	Compound		Inhibitor type	Promastigote IC_{50} value	Amastigote IC_{50} value
1	DMFO	α-Difluoromethylornithine	Irreversible	30 µM	50 µM
2	APA	3-aminooxy-1-aminopropane	Competitive	42 µM	5 µM
3	DAB	1,4 -diamino-2-butanone	NR	144 µM	NR
4	GAPA	γ-guanidinooxypropylamine	NR	40 µM	9±1.0 µM

NR: Not reported

Inhibiting *Leishmania* ODC may inhibit human ODC also, but human protein is resynthesized continuously (*Roberts et al., 2013*). Moreover, structure, kinetic properties, and half-life of the *Ld*ODC are almost different from the human counterparts (*Roberts et al., 2013*).

1.2.7 Transporters of various polyamines: *Leishmania donovani* has transporters for various polyamines (eg. putrescine, spermidine). All transporters have equal affinity for their respective polyamines, and it was reported that uptake of spermidine is seven times higher than putrescine (*Kandpal et al., 1997*). Experimental data proved that transport of putrescine and spermidine occurs via different transporter proteins present in parasite. Moreover, it is also reported that amastigotes and promastigotes use different transporters for uptake of polyamines (*Müller et al., 2001*). Transport of polyamines is pH dependent (*Basselin et al., 2000*). *Lm*POT1, a putrescine transporter protein was found and characterised in *L. major* (*Hasne et al., 2005*). Arginine transport in *Leishmania* occurs via *Ld*AAP3 transporter protein (*Darlyuk et al., 2009*). DAB, an inhibitor of ODC, was reported to inhibit transport of putrescine in *Leishmania* promastigote (*Vannier-Santos et al. 2008*).

1.2.8 Drug Targeting Enzymes

There are few unique enzymes and pathways present in *Leishmania* which may be exploited for designing drugs. Following are few enzymes which were successfully used to discover drugs candidates against the parasite using computational or/and experimental approaches.

i. Ornithine decarboxylase (*Heby et al., 2003)*
ii. Spermidine synthase (*Roberts et al, 2001)*
iii. Trypanothione synthase (*Venkatesan et al, 2011)*
iv. Trypanothione reductase (*Colotti et al, 2013)*
v. Glutathione synthesis (*Kapoor et al., 2000)*
vi. Inhibition of mitochondrial respiratory chain complex leads to apoptotic death of *L. donovani* parasite (*Mehta et al., 2004*).
vii. Caspase-like proteases can be a good target which is responsible for apoptotic like cell death of parasite (*Das et al., 2001*).

Protein modelling and crystallization of key proteins helps to identify new drug candidates. Various vital proteins, responsible for parasite survival, were modelled and screened with different ligand databases. Crystallization of the ligand protein complex and some *in vivo* experiments has also been reported. Some of the proteins crystals made so far are listed below in Table 1.4.

Table 1.4: List of leishmanial enzymes involved in redox metabolism and their crystal structures

Sl.no	Enzyme	*Leishmania*	Other organism
1	Ornithine decarboxylase	NR	Human
2	Spermidine synthase	NR	*Plasmodium* (*Dufe et al., 2007*), *Helicobacter pylori* (*Lee et al., 2005*) & *Homo sapiens* (*Wu et al., 2007*)
3	Trypanothione reductase	*L. infantum* (*Ilari et al., 2012*)	*T. cruzi, C. fasciculate*
4	Trypanothione synthetase	*L. major* (*Fyfe et al., 2012*)	NR
5	S-adenosylmethionine decarboxylase	NR	Human S-adenosylmethionine decarboxylase (*Heby et. al., 2003*)
6	Tryparedoxin	NR	*Crithidia fasciculate* (*Alphey et al., 1999*)

NR: Not reported

1.2.9 Available drugs against leishmaniasis: There are many types of diseases exist around the world, which has been categorized into three groups: Global, Neglected and Most neglected diseases (Figure 1.11). Leishmaniasis comes under neglected disease because of its association with poverty. Pharmacological industries were not much enthusiastic to invest money in research of anti-leishmanial drug development as they do not see potential market incentive (*Yamey, 2002*). The treatment of leishmaniasis is mainly based on chemotherapy. There are very few drugs available in the market and having certain disadvantages like, high cost, resistance, toxicity, side effects, poor efficacy, lack of target specificity etc.

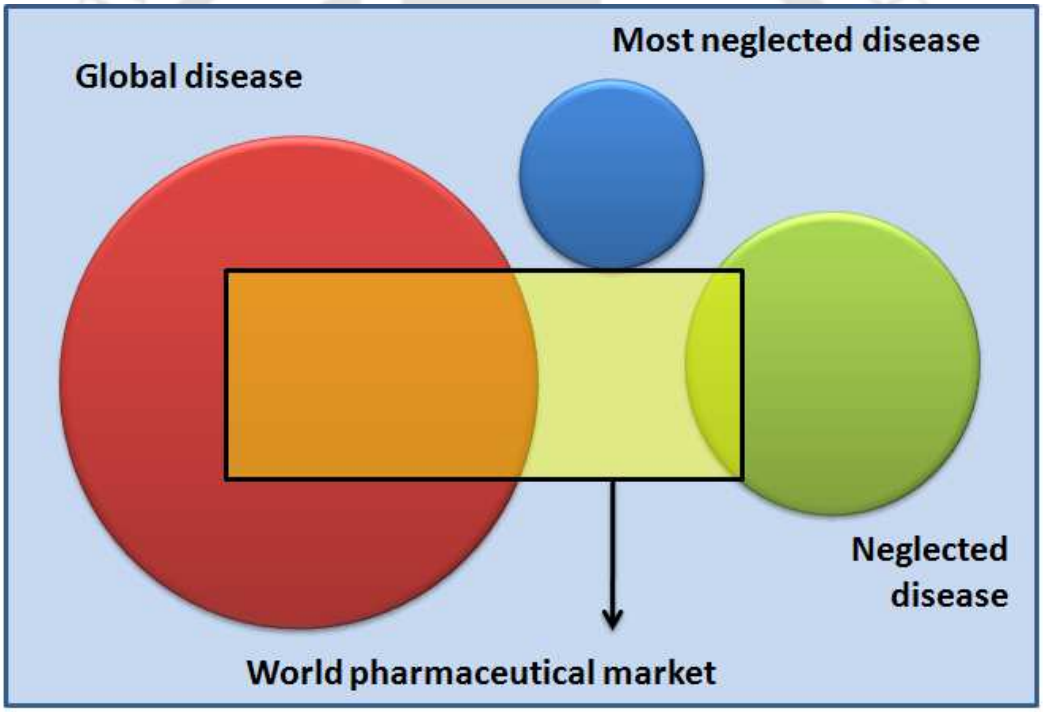


Figure 1.11: There are three major forms (Global, Neglected and Most neglected) of diseases in the world. Leishmaniasis falls under Neglected Disease as it belongs to poor people. Very few scientific communities are involved in studying leishmaniasis. Therefore, very less number of drugs is available against the disease which is depicted from the diagram above (***Source:*** *figure adopted from BMJ, 2002, 325,176–177*).

These limitations of available drugs necessitate development of more and better drug candidates. Various drug candidates were identified by high throughput screening technology and many of them was poorly soluble in water (*Mondal et al., 2013*). The first line of treatment against leishmaniasis is based on the use of pentavalent antimonials like pentostam etc. Other drugs are amphotericin B, paramomycin, fluconazole, ketoconazole, rifampicin, doxorubicin, miltefosine, sitamaquine and azoles. Till now there is no vaccine or a satisfactory chemotherapy available which does not include above mentioned problems. Therefore, there is an urgent need for development of vaccines and effective drugs for the treatment of leishmaniasis.

***Pentavalent antimonials*:** Pentavalent antimonials (sodium stibogluconate, Pentostam, meglumine antimoniate) are the first line of drug for more than six decades. They were introduced in 1940s (*Ashutosh et al., 2007*) and acts by inhibiting glycolysis and oxidation of fatty acid pathway (*Berman et al., 1985*). Sodium stibogluconate inhibits fatty acid β oxidation, glycolysis and inhibits ADP phosphorylation (*Pandey et al., 2009*). There are reports mentioning that the antimonial drugs kill parasite by targeting trypanothione reductase (*Maltezou et al., 2009*). Pentavalent Sb (V) is reduced to trivalent antimony Sb(III) which is the active form of drug (*Roberts et al., 1995*). Reduction occurs in parasite or in macrophage and mechanism of the same is not very well understood. However, it is well documented that trivalent form of Sb is more effective than its pentavalent form (*Roberts et al., 1995*). This drug has side effect ranging from damage to heart, liver, pancreas, hematopoetic tissues as well as local pain at the site of injection (*Arevalo et al., 2001; Soto et. al., 2013*).

***Pentamidine isethionate*:** It is the second line of drug against leishmaniasis which affects polyamine biosynthesis and mitochondrial membrane potentials by attacking kinetoplast DNA (*Pandey et al., 2009; Lindoso et al., 2012*). This drug has many side effects like high toxicity, hypoglycemia, diabetes mellitus, hypotension, myalgia, abscess at the injection site (*Lindoso et al., 2012*).

***Amphotericin B*:** It is the second line of drug since 1960. The drug targets 24th position of substituted sterols mainly ergosterol. It causes aqueous pores resulting in cell lysis (*Pandey et*

al., 2009). This drug was earlier used as antifungal agent and is active against both promastigote and amastigotes. It is also effective against antimony resistant strains. Better efficacy of amphotericin B is achieved with its prolong administration (*Maltezou et al, 2009*). Lipid formulations of amphotericin B has shown 96% cure rate and gives localized action with fewer side effects. The major side effects of the drug includes vomiting, heart problem, renal failure, nausea, fever, nephrotoxic and expensive.

***Paromomycin*:** It is a low cost drug and is administered through intramuscular injection; inhibit protein synthesis and changes membrane fluidity and permeability. It is under phase IV clinical trial (*Pandey et al., 2009*). It posses side effects which are more than amphotericin B and also include pain after injection (*Maltezou et al., 2009*).

***Fluconazole*:** It is a triazole anti fungal agent. It was used for the treatment of cutaneous leishmaniasis which is caused by *L. major, L. braziliensis* (*Blum et al., 2014; Alrajhi et al., 2002; Sousa et al., 2011).* It an oral drug against cutaneous leishmaniasis.

***Ketoconazole*:** It is an antifungal agent and shows 70% cure rate. However, patients with hepatic ailments cannot be treated with ketoconazole for leishmaniasis. (*Blum et al., 2014*)

***Rifampicin*:** It is well tolerated by patients and has no side effects. It is a better alternative drug for oral treatment of cutaneous leishmaniasis (*Kochar et al., 2002*).

***Doxorubicin*:** Initially famous as anti cancerous agent but latter on found to be effective against *Leishmania*; can be considered as second line of drug (*Sett et al., 1992*).

Miltefosine (hexadecylphosphocholine): Miltefosine, a lysophospholipid analog, highly efficient oral drug to be registered in India for the treatment of human visceral leishmaniasis (*Sundar et al., 2002*). It is initially developed as an oral anticancer drug and induces apoptosis in cancer cell lines (*Verma et al., 2004*). The drug has undergone randomised phase IV trial on visceral leishmaniasis patients aged > 12 years, and during that time the long-term cure rate was found to be 94% after 28 days of 50 mg miltefosine twice daily (*Sundar et al., 2000; Sundar et al., 2012*). Miltefosine causes modulation of cell surface receptors, phospholipase activation, inositol metabolism, protein kinase C resulting in apoptosis like cell death (*Pandey et al., 2009*). The drug is useful in T-cell mediated deficient patients.

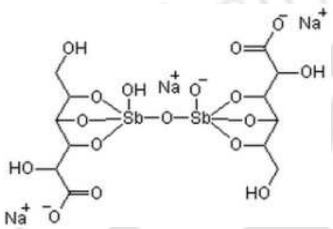
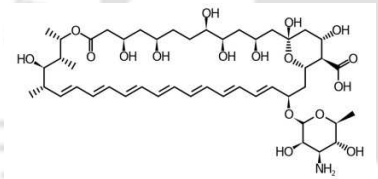
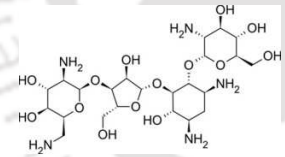
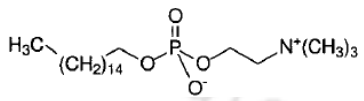
Besides having antifungal, antibacterial, anthelminthic properties (*Eissa et al., 2011*), miltefosine also proved to have *in vitro* ovicidal, schisto-larvicidal and lethal activity on adult worms of both Schistosoma species and has considerable molluscicidal activity on their snail hosts (*Eissa et al., 2011*). Miltefosine is teratogenic and cannot be given to pregnant ladies. Furthermore, it cannot be given to children due to toxic effects as well as gastrointestinal toxicity (*Palit et al., 2012; Maltezou et al., 2009*).

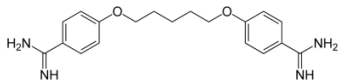
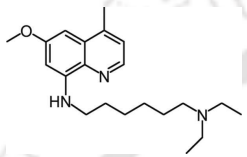
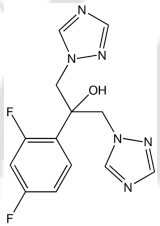
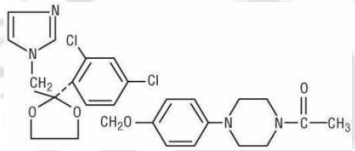
***Sitamaquine (WR-6026)*:** It is also called as 8-aminoquinoline analogue, also acts as antifungal agent and presently in phase II trials (*Pandey et al., 2009*).

***Azoles*:** It inhibits cytochrome P-450–mediated 14 *a*-demethylation of lanosterol and blocks ergosterol synthesis which causes accumulation of 14 *a*-methyl sterols (*Pandey et al., 2009; Alrajhi et al., 2002*)

A comprehensive list of various anti-leishmanial compounds, their structures, current status and targets etc is shown in Table 1.5.

Table 1.5: List of various anti-leishmanial compounds, their structures and current status.

Sl. no.	Drugs	Structure	Side effects	Current status	Drug targets
1	Sodium antimony gluconate (SAG)		Damage to heart, liver, pancreas, hematopoetic tissues, local pain at the site of injection (*Kato et al., 2014*)	First line of drug for more than six decades, under phase IV trial (*Vermeersch et al., 2009, Sinha et al., 2011*)	Ergosterol of membrane, Expel out trypanothione reductase
2	Amphotericin B		Vomiting, heart problem, renal failure, nausea, fever, nephrotoxic and expensive.	Second line of drug since 1960, phase II (*Stuart et al., 2008*)	The drug targets 24th position of substituted sterols mainly ergosterol. It causes aqueous pores resulting in cell lysis (*Cohen, 2010*; *Mbongo et al., 1998*)
3	Paromomycin		Local pain after injection, elevated hepatic transaminases, ototoxicity (*Lindoso et al., 2012*)	Phase IV clinical trial (*Sinha et al., 2011*)	Inhibits protein synthesis by interacting with ribosomal RNA subunits
4	Miltefosine		Toxic to gastrointestinal, hepatic and renal systems. Teratogenic (cannot be given to pregnant ladies) (*Lindoso et al., 2012*)	First line treatment, phase IV trial (*Rahman et al., 2011*)	Inhibits phopholipid and sterol biosynthesis.

5	Pentamidine		Nephrotoxicity, heart problem, low blood pressure, low or high blood sugar (*Silva et al., 2013*)	Second line treatment	Inhibitor of arginine transport, putrescine and spermidine transport (*Basselin et al., 2002*)
6	Sitamaquine		Vomiting, Abdominal pains, Headache, nephrotoxicity, Methemoglobinemia (*Jha, 2006*)	Phase II trials (*Jha, 2006*)	Morphology alteration of *Leishmania,* Rapid collapse of the mitochondrial inner-membrane potential (*Loiseau et al., 2011*)
7	Fluconazole		Mild nausea, cheilitis (*Khan et al., 2014*)	Phase III trails	Obstruct *Leishmania* growth by inhibiting cytochrome P-450-mediated 14-a demethylation of lanesterol, blocking ergosterol synthesis, accumulation of 14-a-4methyl sterols (*Sousa et al., 2011*)
8	Ketoconazole		Nausea, vomiting, stomach pain, and diarrhea	Ketoconazole cream is effective for Cutaneous *leishmaniasis*	Interfere with sterol biosynthesis (*Momeni et al., 2003*)

1.2.10 Immunoresponse: Visceral patients respond by gamma interferon (IFN-ϒ), interleukin 12 (IL-12) (*Maltezou et al., 2009*), cytokines and neutrophils (*Maltezou et al., 2009*) for clearing of the parasite (*Pandey et al., 2009*). $CD4^+$ T-cell causes interferon (IFN)-ϒ-induced macrophage activation (for killing the parasite) through participation of cytokines, mainly IL-12, IL-2, tumor necrosis factor, and also deactivation of macrophage leads to suppression of Th1 responses (*Maltezou et al., 2009*). IFN-ϒ production leads to induction of nitric oxide synthase by macrophages which produce nitric oxide (NO) to kill the parasite. After parasite infection, macrophage mediated innate immune system generate O_2^- and nitric oxide (*Murray et al, 1999*). O_2^- generation does not require previous infection but nitric oxide generation requires activation of macrophage. Younger patients are more susceptible to relapse due to the lack of a mature immune system (*Kajaia et al., 2011*). Induction of T-helper type 2 (Th2) suppressive cytokines (IL-4, IL-13) and/or IL-10 can lead to development of disease (Figure 1.12) (*McConville et al., 2011; Jha, 2006)*. Elevated levels of IL-10 have been seen in VL patients and drug targeting IL-10 can leads to activation of Th1 responses to cure the disease (*Maltezou, 2010*).

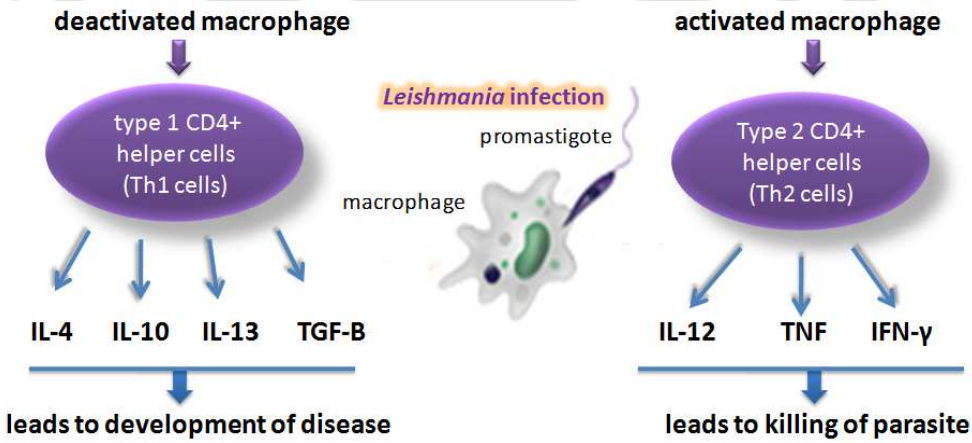


Figure 1.12: *Leishmania* infection may lead to prolong disease or may lead to death of parasite, this all depends on macrophage response, if activated then it kills parasite by activation of certain cytokine and if macrophage is deactivated then it leads to progress of the disease which makes the patient even more worst.

Immunotherapy is also a good kind of treatment, where drugs are combined with antigens, adjuvants, inteferon-γ, other cytokines and inflammatory response modulators, where cure rate may reach upto 90% (*Lindoso et al., 2012*).

1.2.11 Vaccines against *Leishmaniasis*: Vaccines against leishmaniasis have started when certain tribes expose their stomach for sandfly bites in order to avoid facial lesions. Certain tribes also used to transfer infected material from lesion to uninfected individuals by using thorns (*Handman, 2001*). *L. donovani* infected macrophages stimulates MHC class II (MHC-II) restricted T cells after 6 hours of infection. Live attenuated vaccine is a good option but it has safety issues. Heat killed (autoclaved) *Leishmania* was used as a vaccine, which reduced cutaneous leishmaniasis by 18-70% (*Kumar et al., 2014*). List of different types of vaccines used for leishmaniasis and their mode of actions are listed in Table 1.6

Table 1.6: List of different types of vaccines used for leishmaniasis and their mode of actions

S. No	Vaccines	Mode of action	References
1	Heat killed (autoclaved) *Leishmania*	cell-mediated and humoral response	*Nagill et al., 2009*
2	DNA vaccines	humoral and lympho-proliferative immune responses	*Guha et al. 2013* *Dumonteil et al., 2003*
3	CpG motifs from bacterial DNA	Activates B-cells & dendritic cell response	*Handman 2001*
4	Cytokines	Activates Th1 immune response	*Cummings et al., 2010*
5	Adjuvant	humoral or cellular immune response	*Mutiso et al., 2010*
6	Synthetic peptides	innate immunity to infection	*Erfe et al., 2011*
7	Live-Attenuated Vaccines	protective immune responses	*Evans et al., 2012*

The WHO 2010 recommendations for control of leishmaniasis are mainly written for endemic countries (Control of the Leishmaniases, Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22–26 March 2010; 949). In 2014,

LeishMan (Leishmaniasis Management), a group of experts from 13 institutions in eight European countries recommended for treatment of cutaneous and mucosal Leishmaniasis for travelers based on clinical trials between 1960-2013 (*Blum et al., 2014*).

1.2.12 Anti-leishmanial Peptides: Prokaryotes, fungi, plants, amphibians and animals secrete certain molecules to protect themselves from environmental noxious microorganisms (*Kückelhaus et al., 2009*). These secretions are mainly peptides to defend against bacteria, fungi, protozoa, tumour cells and viruses (*Kückelhaus et al., 2009*). These natural antimicrobial peptides now considered to be a good alternative therapeutic option for the control of leishmaniasis (*Eaton et al., 2014*). A List of ant-leishmanial peptides are shown in Table 1.7.

Table 1.7: List of anti-leishmanial peptides used against leishmaniasis and their mode of actions.

S.No.	Anti-leishmanial peptide	Species	Reference
1	RP-1 & AA-RP-1	*L. braziliensi*, *L. infantum*, *L. chagasi*, *L. major*	Erfe et al., 2011
2	Dermaseptin 01 (DRS 01)	*L. infantum*	Eaton et al.,2014
3	Phylloseptin-1 (PS-1)	*L. amazonensis*	Kückelhaus et al., 2009
4	Temporin A, B and 1Sa	*L. maxicana*	Chadbourneet al., 2011
5	Human neutrophil peptide-1 (HNP-1)	*L. major*	Dabirian et al., 2013
6	Cathelicidin bovine myeloid antimicrobial peptide (BMAP-28)	*L. major*	Lynn et al., 2011

For the first time synthetic peptides (RP-1 and AA-RP-1) have been used against *Leishmania* species, it is designed based on prior structure-function knowledge of mammalian microbicidal peptides (*Erfe et al., 2011*). They have minimal toxicity to mammalian cells. Most of them are antimicrobial and anti-malarial also.

1.2.13 Drug resistance in *Leishmania:* Drug resistance is a major challenge for the control of infectious disease like leishmaniasis. List of various strains of *Leishmania* is mentioned in Table 1.8 which has become resistant to certain drugs. Initially drug resistance reports came first for malaria due to misuse of drug. There could be a variety of reasons leading to the development of drug resistance: (i) overproduction of target enzyme which may deplete the concentration of drug, (ii) exclusion of drug or reduce uptake of drug or (iii) conversion of drug into inactive form by an enzyme, (iv) bypass of inhibited pathway by an alternate pathway, (v) alternation of membrane permeability which does not allow drug to pass through it, (vi) Use of drug combinations may lead to resistance (*Bryceson et al., 2001*). Membrane enriched protein's proteomic analysis of *L. donovani* was done recently (*Kumar et al., 2015*). This will help to understand insight and comparison of resistant strain. *Leishmania* is a diploid organism but very few reports are there to support the fact rather several strains proved, *Leishmania* to be an aneuploid.

Sodium stibogluconate was highly effective against leishmaniasis but later, certain species of *Leishmania* have developed resistance against it (*Singh et al., 2014*; *Mukherjee et al., 2007*; *Rai et al., 2013*). This has discontinued the use of antimonials and has led to the employment of amphotericin B against leishmaniasis. The drug was earlier used as antifungal agent, but later it was found effective against *Leishmaniasis*. Resistant strains of amphotericin B were found in Bihar and this resistant strain requires eight fold higher lethal doses (*Purkait et al., 2012*). Purkait *et al* (2012) reported recently that the absence of ergosterol in the amphotericin B resistant *Leishmania*'s membranes, and up-regulated drug's efflux and effective ROS scavenging machinery leads to a cumulative effect in conferring resistance against Amphotericin B.

Miltefosine was effective against antimony resistant strain (*Sundar et al., 2002*) but later, the parasite became resistant to it as well. Then paromomycin came into picture. Due to some adverse effects (elevated hepatic transaminases, ototoxicity, and pain at the injection site) paromomycin is not very popular. Clinically isolated strains of *L. donovani* have shown different susceptibility and antimonial drug resistance varies based on genetic background (*Decuypere et al., 2012*). Sometimes in vitro active compounds are not effective for *in vivo* activity, due to poor pharmacokinetics (*Schmidt et al., 2012*). There is a report that

promastigotes and amastigotes resistance to one drug combination shows cross-resistance to other anti-leishmanial drugs as well (*Garcia-Hernandez et al., 2012*). D,L-alpha-difluoromethylornithine (ODC inhibitor) resistant strain of *Leishmania* becomes susceptible to other inhibitors of same pathway (*Mukhopadhyay et al., 1996*). A report tells that whenever a new drug comes, number of cases and deaths goes down but the parasite gains resistance after few years resulting in increase in number of cases again. Recent combinatorial theory has brought a new hope which helps in resistant cases (*Muniaraj, 2014*).

Table 1.8: List of *Leishmania* resistant strains which has gained resistance towards certain drugs

	Drug	Strain	References
1	Miltefosine	*L. donovani*	*Perez-Victoria et al., 2003; Seifert et al., 2003*
2	Antimony	*L. donovani*	*Singh 2006; Mukherjee et al., 2007; Rai et al., 2013, Purkait et al., 2012*
3	Paromomycin	*L. aethiopica*	*Maarouf et al., 1998; Croft et al., 2006*
4	Pentamidine	*L. donovani, L. amazonensis*	*Rai et al., 2013 Croft et al., 2006*
5	Amphotericin B	*L. donovani*	*Purkait et al., 2012*
6	DFMO	*L. donovani*	*Mukhopadhyay et al., 1996*
7	Meglumine antimoniate	*L. infantum, L. tropica*	*Faraut-Gambarelli et al., 1997 McConville et al., 2011*
8	Arsenite, buthionine sulfoximine	*L. tarentolae*	*McConville et al., 2011*
9	Glucantime	*L. tropica*	*Hadighi et al.,2006*

1.2.14 Miltefosine Resistance of *Leishmania:* Efficacy of miltefosine went down in India after a decade of use due to drug resistance (*Sundar et al., 2012*). Leishmaniasis has gained resistance against miltefosine. Resistant strain is fifteen times more resistant than wild type

strain and remains resistant up to twelve weeks in miltefosine deficient media (*Seifert et al., 2003*). Development of drug resistance against miltefosine could be because of several reasons: (i) long half life, approx 150 hrs (*Maltezou et al., 2009*), (ii) Alterations in fatty acid and sterol biosynthesis (mainly *Leishmania* acyl-CoA synthetase and desaturase systems) resulted in changes in the lipid composition of membranes which alters membrane fluidity and permeability, this may affect drug-membrane interactions (*Rakotomanga et al., 2005*), (iii) defective inward translocation of the drug in resistant strain (*Pérez-Victoria et al., 2003*), (iv) single point mutations in transporter proteins, L832F which leads to decreased uptake of drug (*Pérez-Victoria et al., 2006; Seifert et al., 2007*). (v) According to a report, over-expression of mitochondrial iron superoxide dismutase-A (*Ld*FeSODA) has been found in resistant strain which protects parasites from oxidative stress caused by miltefosine (*Getachew, 2012*). Prolonged incubation of parasites with miltefosine results in the release of *Ld*FeSODA, cytochrome c oxidase, mitochondrial dysfunction further leading to causing apoptotic death (*Verma et al., 2007*; *Luque et al., 2007*). (vi) Over-expressed efflux pumps (*Luque-Ortega et al., 2012*); (vii) The mechanism of miltefosine resistance in *Leishmania* mediated through the redox system seems to be an adaptive change in the parasites resulting due to the indiscriminate use of drugs. Recently effect of metabolic changes in miltefosine-resistant *L. donovani* at the global gene expression level has been studied and explored (*Das et al., 2013*). Growth of miltefosine-resistant *L .donovani* (R40 strain) were inhibited by constructs consisting of an ω-thiol-functionalized miltefosine analogue conjugated to the cell-penetrating peptide (CPP) Tat(48–60), either through a disulfide or a thioether bond (*Luque-Ortega et al., 2012*).

1.2.15 Antimony Resistance of *Leishmania:* Antimony based drugs are first line drugs. Pentavalent Sb (V) reduced to trivalent antimony Sb(III) which is the active form of the drug. Reduction take place either in parasite or in macrophage is still not answered. Pentavalent Antimony (V) resistance was reported on 1981 in Bihar (*Sundar et al., 2002*). Sb (V) is not sensitive towards promastigotes and axenic amastigotes even upto 64 µg/ml concentrations (*Vermeersch et al., 2009*). Antimony resistant strain has shown over-expression of ornithine decarboxylase, γ-glutamylcysteine synthetase, MRPA transporter and down regulation of

uptake transporter aquaglyceroporin, AQP1 (*Mukherjee et al., 2007*; *Rai et al., 2013*). RNA level and enzyme activity of Trypanothione reductase has been seen to increase in resistant strain (*Rai et al., 2013*). Thiol level was also found to be elevated (*Rai et al., 2013*). When Sb targets $T(SH)_2$, then it makes complex Sb-$T(SH)_2$ and it excludes through MRPA transporter. 20 mg/kg/day for 20 consecutive days have been used (*Soto et. al., 2013*). There is a report on occurrence of antimony resistant strain where arsenic contamination in water has been found (*McConville et al., 2013*). Accumulation of arsenic in lever leads to appearance of unresponsive strain of parasite because arsenic based drugs were used to treat the disease.

1.2.16 DFMO resistant strain of *Leishmania:* D,L-α-difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase (*Meyskens et al., 1999*). Earlier it was used to treat carcinogenesis. This drug is also used to treat leishmaniasis, IC_{50} dose is 125 µM (*Mukhopadhyay et al., 1996*). DFMO causes parasite killing by elevating nitric oxide concentration in macrophage infected with parasite. DFMO led to increased uptake of putrescine within promastigotes; therefore DFMO treatment should be accompanied by other inhibitors of same pathway (*Roberts et al., 2013*). DFMO resistant strain shows 15 fold higher ODC activities than wild type and IC_{50} value more than 10 mM (*Hanson et al., 1992*). There are certain side effects related to the drug: seizures, fever, infections, neutropenia, hypertension, diarrhea and septic shock occur.

1.2.17 Co-infections with HIV: Co-infection of HIV in the visceral form, there is an absence of a specific T-cell mediated immune response and mutual exacerbation of infection. There are 1000 cases till date reported from 25 countries showing co-infection (*Vector Borne Disease Control Programme, 2014*). Patients shows low CD4 counts (<50 CD4 cells/mm^3) (*Sundar et al., 2002*). Sensitivity towards antibody-based immunologic tests like the IFA test and ELISA is low for HIV patients (*Sundar et al., 2002*). Antigen detection test is prefered than antibody detection due to low antibody production (*Sundar et al., 2002*).

1.2.18 Animal Models used for *in vivo* Assays: Animal models are supposed to mimic the pathological behaviour of human caused by infections as they all come under mammalian category. So it is better to test in animals first before going for clinical trials, as they provide clues to toxicity, route of administration of drug, absorbtion of drugs, etc. Few animals are proved to be good model for in vivo assays like: (i) Mice (BALB/c mouse), hamster (for primary tests), (ii) dogs (secondary tests), (iii) monkey (Indian langur), vervet, squirrel (tertiary tests) (*Kamhawi, 2006*). They are used because of better availability and low amount of drugs needed.

1.2.19 Combinatorial Therapy: Drug resistance problem has become a severe issue. All the successful existing drugs become ineffective after few years of treatment, but use of drug combination has solved the problem of resistance (Figure 1.13). Combination of known anti-leishmanial agents may help to solve the resistance problem because it will have two to three different targets and this is hard to gain resistance by the parasite. Moreover there are some reports on complete clearance of parasite after undergoing drug combination therapy.

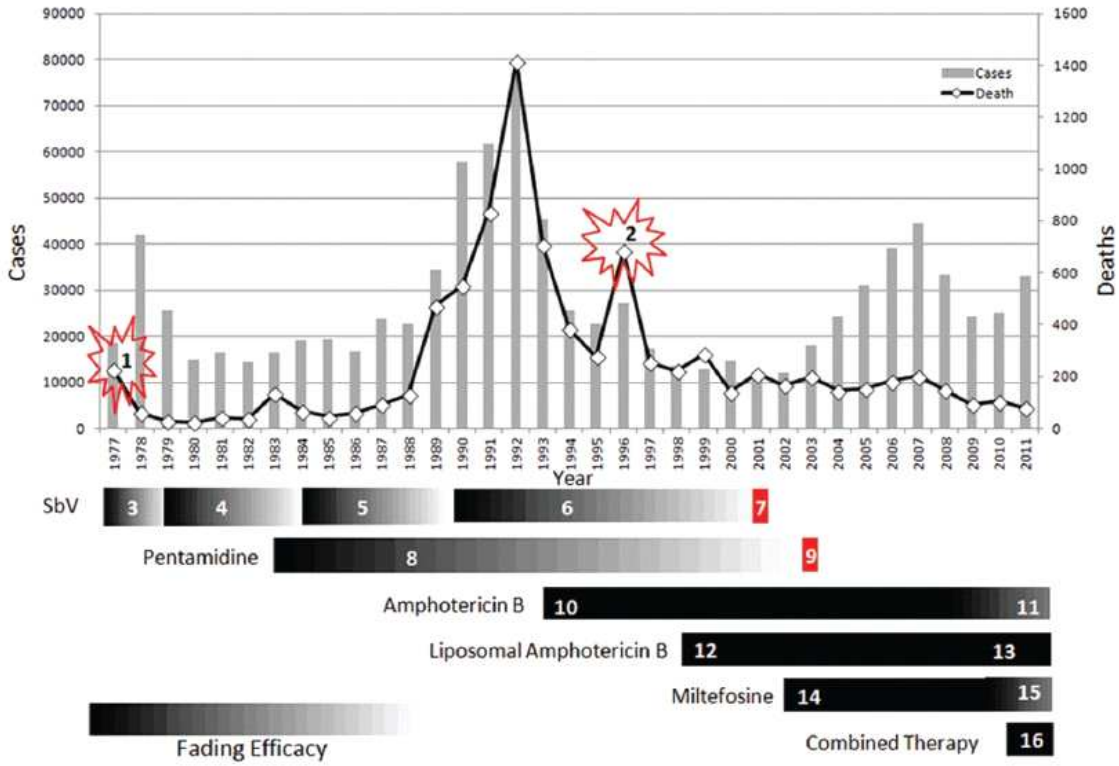


Figure 1.13: New drug decreases number of cases and deaths, but with the fading of efficacy and emergence of drugs resistance, numbers of cases again arise. Combined therapy seems to solve the problem of drug resistance as it is clearly visible in the above graph (***Source:*** figure adopted from *Tropical Parasitology, 2014*, 4, 10–19).

1.3 Significance and scope of current research: Leishmaniasis is an infectious disease caused by a protozoan parasite spread by blood feeding vector (sandfly). Only few drugs are available in market with limitations in terms of side effect, drug resistance or toxicity. Drug resistance is a major global health threat against leishmaniasis, which dissolves the efforts taken for discovery of successful drugs. There could be many reasons behind drug resistance mechanism. For my PhD work one of my objectives is to study the mechanism behind drug resistance against miltefosine. We tried to elucidate the drug resistance mechanism of *Leishmania* by studying miltefosine resistant strain as a model system. We compared gene expression profile of miltefosine-responsive and miltefosine-unresponsive strain of *L. donovani* by microarray. Further, biochemical studies were also performed to experimentally validate microarray data. We reported experimental data that explains the relationship between parasite defense against reactive oxygen species and *Leishmania* resistance to miltefosine. We found that miltefosine-unresponsive strain is better at resisting oxidative stress. Furthermore, we found higher super oxide dismutase and ascorbate peroxidase activity in the resistant strain. Miltefosine and many other drugs against *Leishmania* cause redox imbalance in the parasite. The data suggests that the mechanism of miltefosine resistance in *Leishmania* mediated through the redox system seems to be an adaptive change in the parasites resulting from the indiscriminate use of drugs.

Along with understanding drug resistance mechanism, it is also very important to discover new drugs because *Leishmania* always gains resistance against existing drugs so there is a continuous need of new drugs (Figure 1.14). Unique biochemical and metabolic pathways are found in *Leishmania* which are not present in human counterparts and those novel pathways and enzymes can be exploited for targeting drugs. Therefore, we have chosen ornithine decarboxylase for our study which is proved as drug target. The enzyme is involved in maintenance of redox homeostasis and is a key enzyme in polyamine biosynthesis which converts ornithine to putrescine in *Leishmania donovani*. Further, using integrated computational and biochemical methods, we have identified three novel inhibitors of *Ld*ODC. The *in silico* identified inhibitors were tested on recombinant enzyme. The effect of inhibitors were analysed on the parasite. Our studies suggest that the parasite resists these

*Ld*ODC inhibitors by over-expression of spermidine synthase in *Leishmania donovani* (MHOM/IN/2010/BHU1081).

Figure 1.14: Drug resistance problem can be solved by (i) understanding drug resistance mechanism, (ii) discovery of new novel drug candidates and enzymes (iii) use of combination of known anti-leishmanial agents

Further, computational analysis indicates that *Ld*ODC has unique N-terminal extension which is absent in ornithine decarboxylase of human as well as other organism. *Ld*ODC shows significant sequence and structural difference with the human ornithine decarboxylase. We attempted to explore the role of N-terminal extension which is present in *Ld*ODC. We have cloned, expressed and purified full length as well as truncated (without N-terminal extension) form of the enzyme. Detailed biochemical and biophysical characteristics of the enzyme (full length and truncated form) were analysed to understand role of N-terminal extension. The data suggested that the N-terminal extension helps in folding of the

enzyme in active conformation at the cost of activity, and this is a good example of activity stability trade off.

Additionally, in this report we have employed combinatorial therapy using combinations of various known anti-leishmanial agents that are more effective approach to tackle drug resistance process. To my knowledge and belief that all these three approaches can be beneficial to combat leishmaniasis and throw some light and sprinkle new hopes against leishmaniasis.

The work embodied in this thesis is divided into the following chapters containing the following specific objectives:

I. Studies on miltefosine-unresponsive *L. donovani*: understanding role of redox metabolism in miltefosine resistance:
We tried to understand the underlying mechanism of miltefosine resistance of miltefosine-unresponsive strain of *L. donovani* by performing global gene expression profiling and some biochemical studies.

II. Expression, purification and biochemical studies on ornithine decarboxylase, an important enzyme involved in redox metabolism of *L. donovani* to explore possible role of N-terminal extension:
We have done cloning, expression and purification of *Ld*ODC and T-*Ld*ODC. We did biochemical characterization (pH and temperature optima) and stability comparison of the two forms of ODC. Interestingly we found T-*Ld*ODC is more stable than *Ld*ODC.

III. Novel inhibitors of ornithine decarboxylase of *Leishmania* parasite (*Ld*ODC): The parasite resists *Ld*ODC inhibition by over-expression of spermidine synthase:
We screened drug candidates against *Ld*ODC by doing *in silico* docking method and finally experimentally validating them by doing inhibition studies on purified *Ld*ODC enzyme. We have calculated inhibition parameters like *Ki* value and IC_{50} values. We have explored physiological effects of best selected compounds on *Leishmania*

promastigotes by ROS analysis, estimated polyamine levels, gene expression levels etc.

IV. Effects and response of different drug combinations on the parasite: exploring a combinatorial therapy:

Combination of miltefosine and PS-203 has shown depletion of growth in miltefosine resistant strain. Promastigote viability further PS-203 has already shown a good inhibition on trypanothione reductase enzyme and promastigote growth.

CHAPTER II

Miltefosine unresponsive *Leishmania donovani* has better ability of resist reactive oxygen species.*

2.1 Abstract

Most of the available drugs against Leishmaniasis have limitations related to drug resistance. Resistance of *Leishmania* parasites to miltefosine, which is only available oral drug, is a great concern. The mechanism of miltefosine resistance is poorly understood and only limited studies have been reported in the literature. We have analyzed global gene expression profiles of miltefosine-unresponsive and miltefosine-responsive *Leishmania donovani* in order to understand the various metabolic processes involved in miltefosine drug resistance. The microarray data clearly indicated a role of oxidative metabolism in miltefosine resistance. Furthermore, fluorescence microscopy experiments suggested that miltefosine-unresponsive *Leishmania donovani* resists the accumulation of reactive oxygen species and subsequent mitochondrial membrane damage leading to apoptotic death or programme cell death. In contrast, in miltefosine-responsive *Leishmania donovani*, the accumulation of reactive oxygen species causes apoptotic death. Overall, this study provides fundamental insights into miltefosine resistance in *Leishmania donovani*.

Keywords: Ornithine decarboxylase; Leishmaniasis, inhibitors.

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2.2 Introduction

The only available oral drug against human visceral leishmaniasis is miltefosine (hexadecylphosphocholine), a lysophospholipid analog that has been suggested as a potential oral treatment for human visceral leishmaniasis, structure shown in figure 2.1 (*Sundar et al., 2002*). Miltefosine was primarily developed as an anticancer drug, and it was found that the drug induces apoptosis in various cancer cell lines by targeting cell membranes (*Leonard et al., 2001; Ruiter et al., 2003*). Subsequent studies have reported that miltefosine also causes apoptotic death of *Leishmania* parasites. The apoptotic death was mediated through inhibition of cytochrome c oxidase and mitochondrial dysfunction (*Verma et al., 2007; Luque-Ortega et al., 2007*). After emergence of amphotericin B resistance strains, miltefosine was a good hope. It is well tolerated, widely distributed throughout the body and has cured most of the patients. Miltefosine is the first drug for the treatment of cutaneous or mucocutaneous leishmaniasis. It is more effective (induces apoptosis) against arsenite resistant strain (*Verma et al., 2007*) and has DNA degradation capacity. The drug is not too old, discovered in the early 1980s and in mid-1990s, successful clinical trials for miltefosine were conducted. Unfortunately, occurrence of *Leishmania donovani* resistant strain against miltefosine was first reported in elsewhere (*Seifert et al., 2003*). Miltefosine needs long treatment dose and has long half life and this may be the reason why the drug has gained resistance. It has been reported that over-expression of mitochondrial iron superoxide dismutase-A (*Ld*FeSODA) of *Leishmania donovani* protects parasites from miltefosine (*Getachew et al., 2012*). Furthermore, prolonged incubation of parasites with miltefosine resulted in the release of *Ld*FeSODA and cytochrome c, causing apoptotic death. It has been suggested that *Ld*FeSODA protects the mitochondria of *Leishmania* from oxidative stress, thereby inhibiting programmed cell death (*Getachew et al., 2012*).

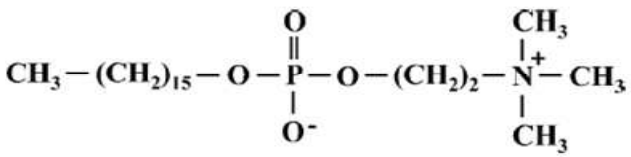

Figure 2.1: Miltefosine is an alkylphosphocholine with a long-chain fatty acid-like backbone of class phospholipids or hexadecylphosphocholine.

There is no report on natural miltefosine resistance with respect to reactive oxygen species (ROS). Furthermore, global gene expression profiles of miltefosine-unresponsive and miltefosine responsive *L. donovani* strains have not been analyzed. The miltefosine transporter *Ld*MT and its specific ß-subunit *Ld*Ros3 form the miltefosine translocation machinery at the *Leishmania* plasma membrane (Figure 2.2) (*Pérez-Victoria et al., 2006*). Miltefosine resistance in *Leishmania* is reported to be caused by single point mutations in these transporter proteins which lead to inactivation (*Seifert et al., 2007; Seifert et al., 2003*). The mutations in miltefosine transporters result in decreased uptake, increased efflux, and faster metabolism of drug (*Pérez-Victoria et al., 2006*; *Seifert et al., 2007*).

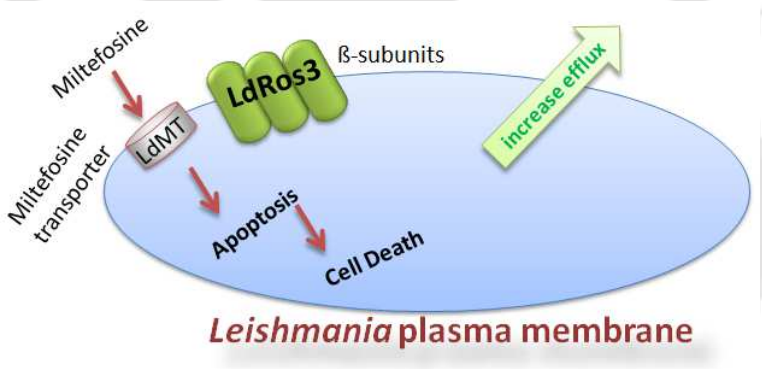

Figure 2.2: The diagram shows one of the reasons behind miltefosine resistance, single point mutation of miltefosine transporter protein which does not allow the drug to enter inside the parasite. Due to this decreased influx, increased efflux and faster metabolism of miltefosine, it fails to cause apoptosis and subsequent death processes.

It has also been reported that miltefosine resistance causes alterations in fatty acid and sterol biosynthesis (mainly *Leishmania* acyl-CoA synthetase and desaturase systems) that result in changes in the lipid composition of membranes of *L. donovani* promastigotes (*Rakotomanga et al., 2005*). Changes in lipid composition of miltefosine-resistant *L. donovani* promastigotes alter membrane fluidity and permeability, and this may affect drug-membrane interactions (*Rakotomanga et al., 2005*). However, the effect of metabolic changes in miltefosine-resistant *L. donovani* at the global gene expression level has not yet been studied. The aim of

the current study was to analyze changes in the gene expression profile of miltefosine-unresponsive *L. donovani* with respect to miltefosine-responsive *L. donovani*. Data from gene expression profiling were experimentally validated by doing some biochemical studies. Our results indicate that miltefosine-unresponsive *L. donovani* accumulates reactive oxygen species less than miltefosine-responsive *L. donovani*. Apparently, miltefosine unresponsive *L. donovani* has better redox machinery that can remove reactive oxygen species more efficiently.

2.3 Materials and methods

2.3.1 Parasites, cell lines, and chemicals Miltefosine-responsive *L. donovani* (BHU-1081) and miltefosine-unresponsive *L. donovani* (BHU-1155) were obtained from Prof. Shyam Sundar (Banaras Hindu University, India), and cultivated in M199 liquid medium supplemented with 15% heat-inactivated fetal bovine serum, 100 U. mL^{-1} penicillin, and 100 µg. mL^{-1} streptomycin. Miltefosine-unresponsive *L. donovani* was obtained from splenic material of a patient suffering from leishmaniasis after a month of miltefosine treatment (18-year-old male with no past history of the disease). All chemicals used were of the highest grade, and were obtained from Sigma-Aldrich or Merck.

2.3.2 Microarray data collection and analysis**:** *L. donovani* microarray (slides, 8 9 15k; Agilent Micro-Array Design Identifier 035638) was used for analysis of miltefosine-responsive *L. donovani* and miltefosine-unresponsive *L. donovani*. Total Ribonucleic acid (RNA) was isolated with the RNAeasy Mini Kit (Qiagen product ID 74104), according to the manufacturer's protocol, and the concentration and purity of the RNA extracted were evaluated spectrophotometerically. The integrity of the extracted RNA was confirmed with a bioanalyzer (Agilent; 2100). We considered RNA to be of good quality on the basis of the 260 nm/280 nm values, rRNA 28S/18S ratios, and RNA integrity number (RIN-Bioanalyzer).

The microarray experiment was performed in collaboration with Genotypic Technology Private Limited (Bangalore, India), according to their optimized procedure

(Figure 2.3). In brief, the samples were labelled by use of the Agilent Quick Amp Kit (Part number: 5190-0442). Five hundred nanograms of total RNA was reverse transcribed with oligodT primer tagged to the T7 promoter sequence. The cDNA (complimentary DNA) thus obtained was converted to double-stranded cDNA in the same reaction. The cDNA was converted to cRNA (complimentary RNA) in the in vitro transcription step, with T7 RNA polymerase, and Cy3 dye (cyanine 3-labelled CTP) was added to the reaction mix. During cRNA synthesis, Cy3 dye was incorporated into the newly synthesized strands (one-colour microarray-based gene expression analysis). The cRNA obtained was cleaned with Qiagen RNeasy columns (Qiagen; Cat. No.74106). Concentration and amount of dye incorporated were determined with the nanodrop technique. Samples that passed the quality control for specific activity were taken for hybridization. Six hundred nanograms of labeled cRNA was hybridized on the array with the Gene expression hybridization kit (Part No. 5190-0404; Agilent) in Sure hybridization chambers (Agilent) at 65 °C for 16 h. Hybridized slides were washed with Agilent Gene Expression wash buffers (Part No. 5188-5327). The hybridized, washed microarray slides were then scanned on a G2565C scanner (Agilent Technologies). Images were quantified with FEATURE EXTRACTION software (Version 10.7; Agilent). Feature-extracted raw data were analyzed with GENESPRING GX Version 12.0 software from Agilent. Normalization of the data was performed in GENESPRING GX 12.0 software, using the 75th percentile shift. Percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted. This normalization takes each column in an experiment independently, and computes the n^{th} percentile of the expression values for this array, across all spots, where n has a range from 0 to 100, and n = 75 is the median. It subtracts this value from the expression value of each entity. Significant genes that were up regulated and down regulated, showing one-fold and above increased expression within the samples with respect to control samples, were identified. For filtering Up regulated genes we consider flag should be “Detected” in the treated samples and can be “Compromised” or “Detected” in the control samples and cut-off used is fold >= 0.6 in fold of all Treated samples and fold >=0.8 in Geomean fold of treated samples. For filtering Down regulated genes we consider flag should be “Detected” in the treated samples and can be “Compromised” or “Detected” in the control samples and cut-off used is fold <= 0.6 in

fold of all Treated samples and fold <=0.8 in Geomean fold of treated samples. A Student's t-test P-value was calculated for the replicate samples. Differentially regulated genes were clustered by using hierarchical clustering based on a Pearson coefficient correlation algorithm, to identify significant gene expression patterns. Functional classification was performed on the basis of gene ontology (GO) functions. Pathway analysis was performed with the KEGG (Kyoto Encyclopedia of Genes and Genomes) database.

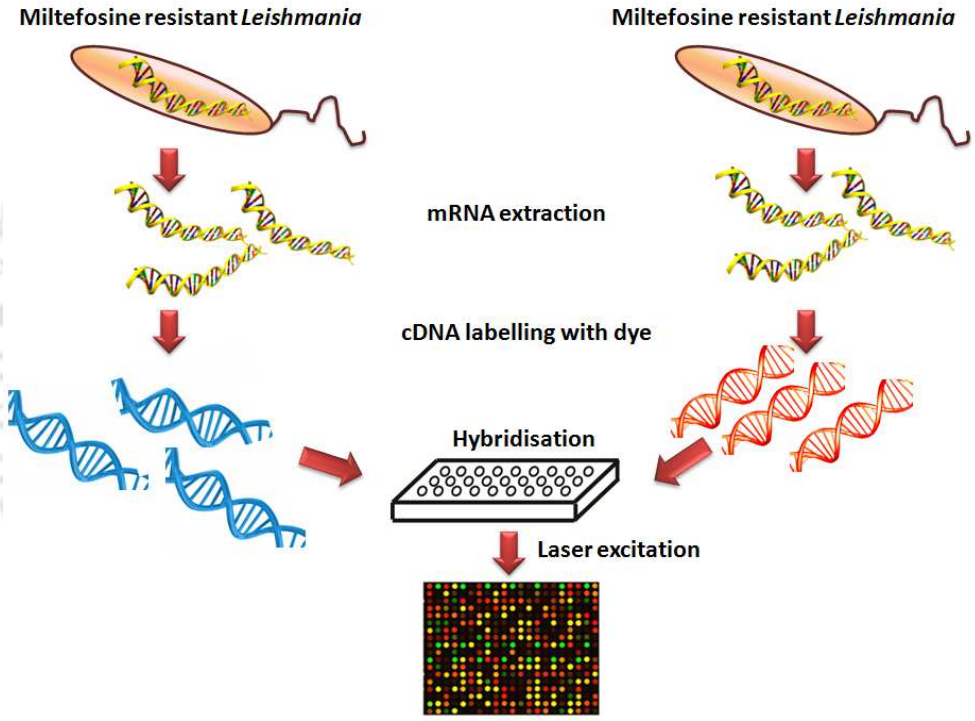

Figure 2.3: Schematic representation of Microarray experiment: Isolation of total RNA from control and tested samples, converted into cDNA by reverse transcription by using oligodT primer. T7 RNA polymerase is used to convert cDNA to cRNA. Cy3 dye is incorporated in newly synthesized strand of cRNA. Labelled cRNA was hybridized on the array with Gene Expression Hybridization kit in hybridization chambers at 65 °C for 16 h. Hybridized slides were washed with Gene Expression wash buffers. The hybridized, washed microarray slides were then scanned on a scanner. Images were quantified with software. Raw data were analyzed with software. ***Source:*** redesigned from available image from web resources

***2.3.3 Generation of reactive oxygen species*:** *Leishmania* promastigotes (2×10^7 cells/ ml) were treated with menadione (10 µM), a naphthoquinone, for three hours to induce reactive oxygen species, as these compounds have been reported to induce reactive oxygen species in the pathogens. Then cells were washed with PBS and suspended in M199 media prior to incubation with 10 µM of a cell-permeable probe, 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (H_2DCFDA) at 25°C for 30 min in the dark. Endogenous reactive oxygen species in *L. donovani* (miltefosine-responsive and miltefosine-unresponsive) were measured with H_2DCFDA by flow cytometry, as reported previously (*Horta et al., 2012; Shukla et al., 2011*). Flow cytometric studies were carried out with a BD FACS Calibur flow cytometer, and analysis was performed with CELLQUESTPRO software (Becton Dickinson).

***2.3.4 Apoptosis Detection by Annexin V-FITC and PI Staining*:** One of the hallmarks of apoptosis is exposure of phosphatidylserine on the cell surface, which can be detected by using FITC-conjugated annexin V. Necrotic cells also bind to annexin V-FITC, but are also stained with Propidium iodide (PI), as membrane damage in necrotic cells allows PI to enter the cell and stain deoxyribonucleic acid (DNA). The apoptosis in *L. donovani* was studied with an annexin V–FITC apoptosis detection kit (Calbiochem). Miltefosine-responsive and miltefosine-unresponsive strains were treated with menadione and miltefosine separately, cells were harvested by centrifugation at 1000 × g for 5 min at 4°C and washed twice with cold PBS. The cells (1×10^7 cells/ ml) were suspended in 500 µl of 1× binding buffer and stained with annexin V-FITC antibody and PI according to the manufacturer's instructions.

***2.3.5 Alteration in mitochondrial membrane potential*:** The mitochondrial transmembrane potential of the parasites was studied by fluorescence microscopy after staining with the MitoCapture apoptosis detection kit (Calbiochem). The kit utilizes MitoCapture, a cationic dye that fluoresces differently in healthy and apoptotic cells. In healthy cells, MitoCapture accumulates and aggregates in the mitochondria, giving off bright red fluorescence. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria, owing to the altered mitochondrial transmembrane potential, and therefore remains in the cytoplasm in its monomeric form, fluorescing green. In brief, promastigote *Leishmania* cells ($1x10^7$ cells mL^-

^{1}) were harvested by centrifugation at 1000 g for 5 min at 4 °C, and washed with cold PBS. The cells ($1x10^{6}$ cells mL^{-1}) were suspended in 100 µL of incubation buffer containing MitoCapture reagent, which was diluted according to the manufacturer's instructions, and incubated at room temperature for 30 min (time optimized for good fluorescence image). After being stained, the cells were washed twice with PBS, mounted on the glass slide, and photographed under a fluorescence microscope (Motic AE31).

***2.3.6 Intracellular Ca^{2+} measurement*:** The fluorescent probe Fura2AM was used to measure intracellular Ca^{2+} concentration (*Gupta et al., 2006*). Briefly, miltefosine-sensitive and miltefosine-insensitive cells that had been subjected to different treatments were harvested and washed twice (500 µL of buffer each time) with wash buffer containing 5.5 mM glucose, 116 mM NaCl (sodium chloride), 0.8 mM $MgCl_2$ (magnesium chloride), 5.4 mM KCl (potassium chloride), and 50 mM MOPS (pH 7.4). Cells were then incubated at 25 °C with 8 µM Fura2AM in the same buffer containing 15% sucrose for 6 hours. Cells were centrifuged (1000 g for 5 min), washed twice, and suspended in the same wash buffer. Fluorescence was measured with excitation and emission at 340 nm and 510 nm, respectively.

***2.3.7 Redox enzyme assay*:** Equal numbers of *Leishmania* promastigote cells (both miltefosine-responsive and miltefosine unresponsive) were taken separately, harvested by centrifugation (1000 g for 5 min), and washed twice with cold PBS. The pellet was dissolved in 1 mL of 20 mM Tris/HCl (pH 7.5), protease cocktail inhibitors, and 1.5 mM $MgCl_2$, and sonicated. After centrifugation, the supernatant was used for enzyme activity assay. Bradford method was used for protein determination and Bovine serum albumin (BSA) was used as standard. Superoxide dismutase (SOD) assay was performed with the riboflavin-mediated Nitro Blue tetrazolium reduction method (*García-Limones et al., 2002*). The total 3-mL volume of SOD reaction mixture contains 13 mM methionine, 75 µM Nitro Blue tetrazolium, 0.1 mM EDTA and 4 µM riboflavin in 20 mM Tris/HCl (pH 7.5) in a glass test tube. All tubes were placed under fluorescent light (13 W) in an aluminum foil-lined box for 15 min, and spectrophotometric reading was performed at 560 nm. The blank and control were

without illumination and without enzyme respectively. One unit is the amount of SOD that inhibits the rate of increase in absorbance resulting from Nitro Blue tetrazolium–diformazan formation by 50%. SOD unit = 50% inhibition = 1 unit. Activity = unit/mg protein.

$$\% \text{ inhibition} = \left(\frac{\text{control} - \text{sample}}{\text{control}}\right) * 100$$

Ascorbate peroxidase (APX) assay was performed as described previously (*Gunes et al., 2009; Singh et al., 2012*). Ascorbate peroxidase assay mixture (1 mL) contains 500 µM ascorbate solution, 10 µM H_2O_2, 100 µM EDTA and 100 µL of *Leishmania* cell lysate in 12 mM Tris/HCl (pH 7.5). The oxidation of ascorbate was monitored by the decrease in absorbance at 290 nm in 3 min, and activity was calculated by the use of the extinction coefficient (2.8 mM^{-1} cm^{-1}).

Formula: A= εcl, where A: absorbance, ε : 2.8 mM^{-1} cm^{-1}, c: concentration, l: cuvette path length in cm. Activity = c/mg protein.

2.4 Results and discussion

***2.4.1 Microarray data analysis*:** The data are plotted in the form of scatter plots and box whisker plots that show acceptable quality (Figure 2.4). In the scatter plot, each dot represents each gene (8064). X-axis represents miltefosine responsive strain and Y-axis is for miltefosine unresponsive strain. The dots lying in middle imaginary line represents those genes which are equally expressed in both the strain. Dots below the middle imaginary line are those genes which were down regulated in unresponsive strain and vice versa. The Box whisker plot presents the distribution of the conditions in the active interpretation with respect to the active entity list in the experiment. The box whisker shows the median in the middle of the box, the 25th percentile and the 75th percentile, or the 1st and 3rd quartile. The whiskers are extensions of the box, snapped to the point within 1.5 times the interquartile. The points outside the whiskers are plotted as they are, but in a different colour and could normally be considered the outliers. The data analysis indicated that 85 genes were up-regulated and 42 genes were down regulated (Figure 2.5). The up-regulated and down-regulated genes were annotated by use of the KEGG database (Table 2.1). The annotated up-

regulated genes are mainly involved in fatty acid metabolism, or encode proteins involved in oxidative metabolism (proteins of oxidative phosphorylation or enzymes involved in ubiquinone synthesis, a component of the electron transport chain). As alteration of fatty acid and sterol metabolism in miltefosine-unresponsive *L. donovani* has already been studied with biochemical methods (*Rakotomanga et al., 2005*), we focused primarily on oxidative metabolism. An efficient electron transport chain plays an important role in keeping the ROS concentration low (*Pramanik et al., 2011*). Our microarray data (accession number GSE45496) suggests up-regulation of genes involved in the synthesis of ubiquinone, an essential component of the electron transport chain, and a few other genes involved in oxidative phosphorylation. Functional classification of gene expression data clearly indicated that genes involved in oxidoreductase activity and redox homeostasis are up-regulated (Table 2.2). Taken together, the data indicated that miltefosine-unresponsive *L. donovani* has more efficient oxidative phosphorylation and a better ability to maintain redox homeostasis.

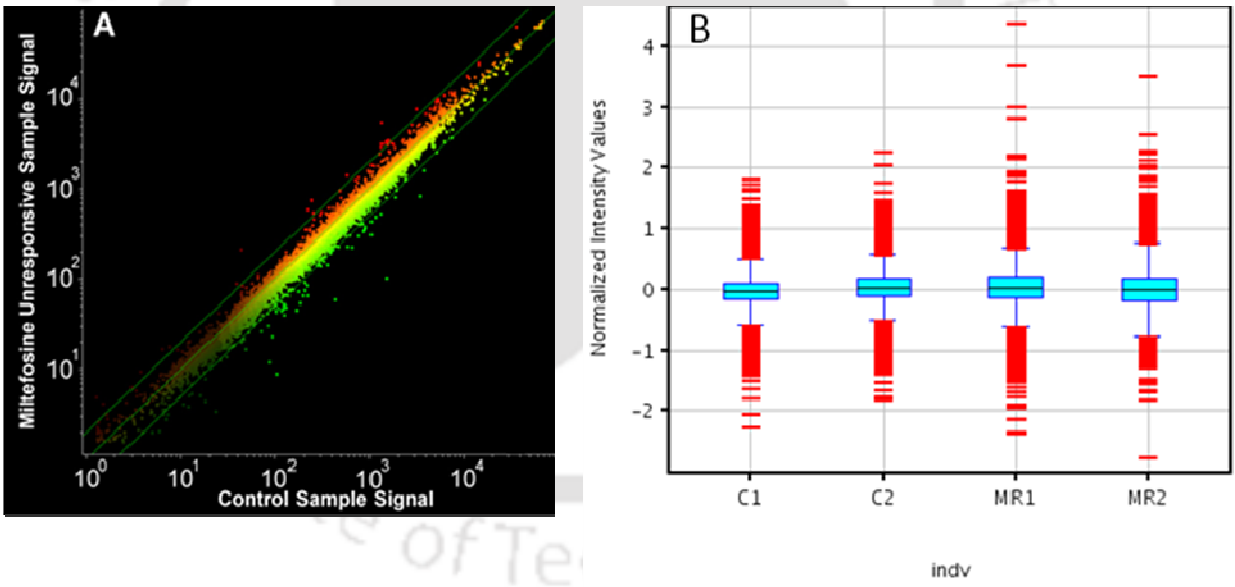

Figure 2.4: Analysis of Microarray data (A) Scatter Plot based on Signal Intensities: A graph of plotted points that show the relationship between two sets of data. Y-axis represents miltefosine resistance and x-axis indicates control. Total 85 genes were found to be up-regulated while 42 genes were down regulated. (B) Box Whisker Plot: another mode of representation of scatter plot. Samples C1 and C2 represent two control experiments while MR1 and MR2 represent two miltefosine unresponsive samples.

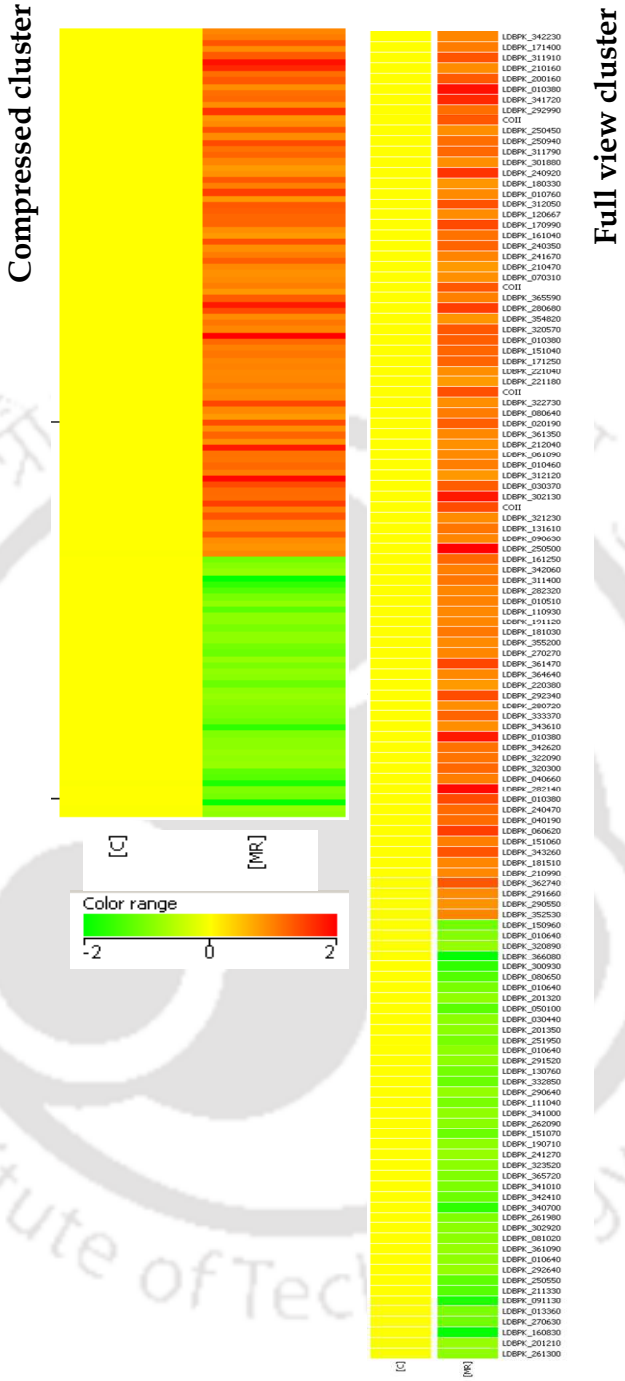

Figure 2.5: Overview of Clusters for Differentially regulated genes: The clusters were generated for the differentially expressed genes taking the Geomean of the control and the Miltefosine unresponsive samples. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns [Clustering algorithm measures the similarity (difference) between genes]. Total 85 genes were found to be up-regulated while 42 genes were down regulated.

Table 2.1: The pathways for the up and down-regulated genes were annotated using KEGG database.

KEGG ID Pathway	Number of genes	Gene Symbol Description
Pathways up-regulated		
ldo01100 Metabolic pathways	4	LDBPK_010510 long chain fatty acid CoA ligase, putative LDBPK_280680 hypothetical protein LDBPK_362740 folylpolyglutamate synthetase LDBPK_365590 ubiquinone biosynthesis methyltransferase, putative
ldo00071 Fatty acid metabolism	1	LDBPK_010510 long chain fatty acid CoA ligase, putative
ldo04146 Peroxisome	1	LDBPK_010510 long chain fatty acid CoA ligase, putative
ldo00190 Oxidative phosphorylation	2	LDBPK_181510 P-type H+-ATPase, putative LDBPK_280680 hypothetical protein
ldo00790 Folate biosynthesis	1	LDBPK_362740 folylpolyglutamate synthetase
ldo00130 Ubiquinone and other terpenoid-quinone biosynthesis	1	LDBPK_365590 ubiquinone biosynthesis methyltransferase, putative
ldo04145 Phagosome	1	LDBPK_280680 hypothetical protein
ldo01110 Biosynthesis of secondary metabolites	1	LDBPK_365590 ubiquinone biosynthesis methyltransferase, putative
ldo03010 Ribosome	1	LDBPK_342620 40S ribosomal protein S19 protein, putative
ldo03008 Ribosome biogenesis in eukaryotes	1	LDBPK_301880 hypothetical protein
ldo03022 Basal transcription factors	1	LDBPK_301880 hypothetical protein
Pathways down regulated		
ldo01100 Metabolic pathways	2	LDBPK_151070 glutamate dehydrogenase LDBPK_190710 glycosomal malate dehydrogenase
ldo00250 Alanine, aspartate and glutamate metabolism	1	LDBPK_151070 glutamate dehydrogenase
ldo00330 Arginine and proline metabolism	1	LDBPK_151070 glutamate dehydrogenase
ldo00630 Glyoxylate and dicarboxylate metabolism	1	LDBPK_190710 glycosomal malate dehydrogenase
ldo01110 Biosynthesis of secondary metabolites	1	LDBPK_190710 glycosomal malate dehydrogenase
ldo00020 Citrate cycle /TCA cycle	1	LDBPK_190710 glycosomal malate dehydrogenase
ldo00910 Nitrogen metabolism	1	LDBPK_151070 glutamate dehydrogenase
ldo03013 RNA transport	1	LDBPK_091130 eukaryotic translation initiation factor 2 subunit, putative
ldo00620 Pyruvate metabolism	1	LDBPK_190710 glycosomal malate dehydrogenase

Table 2.2: Functional classification of gene expression data. Top five up-regulated and down-regulated GO Terms were selected

GO ID	GO Accession/ Gene Ontology Function name	GO Term/Description of Gene Ontology	p-value	Count in Selection	% Count in Selection	Count in Total	% Count in Total
Up-regulated Functions							
8424	GO:0015036	Disulfide oxidoreductase activity	4.05E-06	5	15.15152	27	0.781929
9708	GO:0016667	Oxidoreductase activity, acting on a sulfur group of donors	1.54E-05	5	15.15152	35	1.013611
20728	GO:0045454|GO:0030503|GO:0045867|GO:0045868	Cell redox homeostasis	4.85E-05	5	15.15152	44	1.274254
11673	GO:0019725	Cellular homeostasis	5.42E-05	5	15.15152	45	1.303215
18587	GO:0042592	Homeostatic process	5.42E-05	5	15.15152	45	1.303215
Down-regulated Functions							
3809	GO:0005509	Calcium ion binding	2.99E-04	4	21.05263	62	1.79554
19582	GO:0043648	Dicarboxylic acid metabolic process	7.88E-04	2	10.52632	8	0.231683
2965	GO:0004352	Glutamate dehydrogenase activity	5.50E-03	1	5.263158	1	0.02896
4270	GO:0006103	2-oxoglutarate metabolic process	5.50E-03	1	5.263158	1	0.02896
4273	GO:0006106	Fumarate metabolic process	5.50E-03	1	5.263158	1	0.02896

p-value represents probability of obtaining the specified GO accession number from a list of random entities. Less the p-value more significant is the GO accession number. Count in selection refers to the number of genes in the selected entity (for example, from T-test) list which have that particular GO term. Percentage count in selection refers to the percentage of genes in the input entity list which have that GO term. Count in total refers to the number of genes in all Entities which have that GO term. Percentage count in total refers to the percentage of genes in all entities which have that GO term.

***2.4.2 Detection of ROS*:** Parasites were treated with menadione (10 µM), a naphthoquinone, for 3 hours to induce ROS, as these compounds have already been reported to induce ROS in the pathogen (*Getachew et al., 2012; Alzate et al., 2007*), and flow cytometric analyses were performed. The data clearly indicated that miltefosine responsive *L. donovani* (BHU-1081) generated more ROS than miltefosine-unresponsive *L. donovani* (BHU-1155) under similar menadione treatment. Furthermore, when miltefosine-responsive *L. donovani* was treated with miltefosine (25 µM), there was significant ROS generation, which is consistent with previous studies (*Moreira et al., 2011*). Interestingly, miltefosine-unresponsive *L. donovani* did not generate significant ROS after similar treatment with miltefosine. In all cases where ROS were generated, pre-treatment with N-acetylcysteine (NAC), a scavenger of ROS, was able to remove ROS, ruling out any experimental artefact (Figure 2.6).

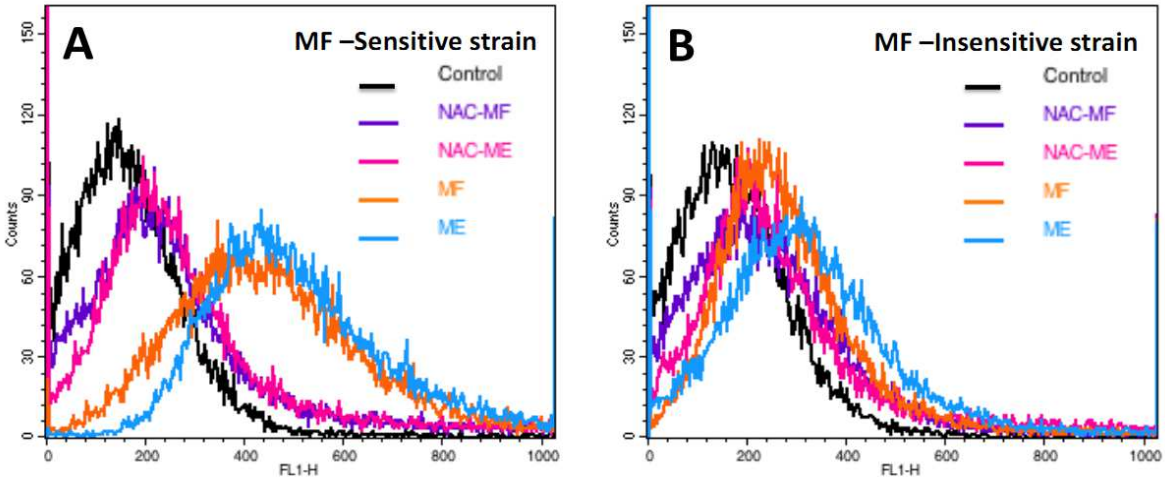

Figure 2.6: Miltefosine-induced and menadione-induced ROS were measured by 2', 7'-dichlorofluorescein diacetate fluorescence, as assayed with flow cytometry. (A) Miltefosine-responsive *L. donovani*. (B) Miltefosine-unresponsive *L. donovani*. Control represents *L. donovani* promastigotes without any treatment; MF and ME represent miltefosine-treated and menadione-treated *L. donovani* promastigotes, respectively. NAC-MF and NAC-ME represent *L. donovani* promastigotes pre-treated with NAC before miltefosine and menadione treatment, respectively. The data shown are representative of at least three experiments that gave similar results.

***2.4.3 Apoptosis Detection by Annexin V-FITC and PI Staining*:** Apoptotic assays indicated that miltefosine-responsive *L. donovani* undergoes apoptosis [increased propidium iodide

(PI) and annexin V–fluorescein isothiocyanate (FITC) fluorescence] after menadione (10 μM) or miltefosine (25 μM) treatment. However, NAC pre-treatment before menadione or miltefosine treatment prevented apoptosis, suggesting a clear link between ROS species and the apoptotic process. Additionally, when miltefosine-unresponsive *L. donovani* was treated similarly with menadione or miltefosine, no apoptosis was seen (Figure 2.7).

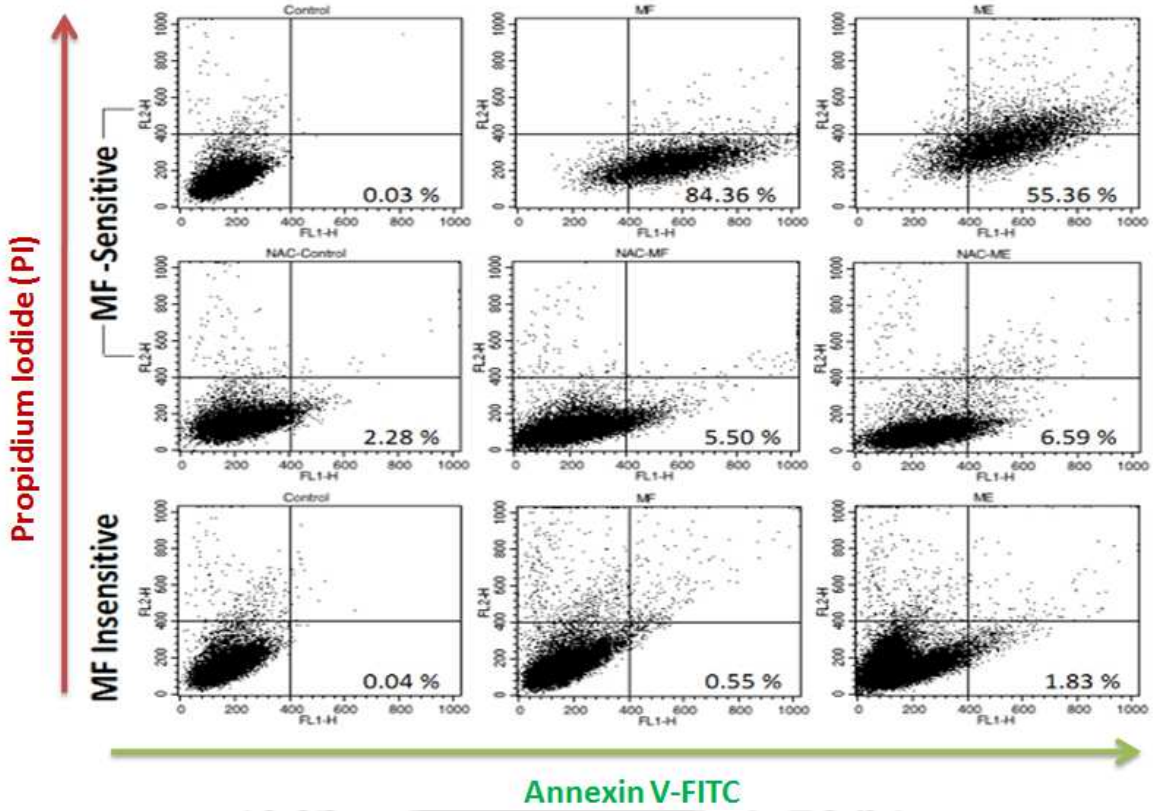

Figure 2.7: Flow cytometric analysis. *L. donovani* promastigotes treated with miltefosine or menadione for 6 h were double stained with PI and annexin V–FITC. Untreated *L. donovani* promastigotes after staining with PI and annexin V-FITC was used as control. MF and ME represent miltefosine-treated and menadione-treated *L. donovani* promastigotes, respectively. NAC-MF and NAC-ME represent *L. donovani* promastigotes pre-treated with NAC before miltefosine or menadione treatment, respectively. The FL1 signal (x-axis) indicates the annexin V–FITC fluorescence value, and the FL2 signal (y-axis) represents the PI fluorescence value. The data shown are representative of at least three experiments that gave similar results.

***2.4.4 Alteration in mitochondrial membrane potential*:** We observed a clear correlation between mitochondrial membrane damage, apoptosis and ROS generation in miltefosine-responsive *L. donovani*, more ROS being generated than in miltefosine-unresponsive *L. donovani*. After menadione (10 μM) or miltefosine (25 μM) treatment, miltefosine-responsive *L. donovani* showed disruption of mitochondrial membrane potential. Pre-treatment with NAC before similar treatment with menadione or miltefosine in miltefosine-responsive *L. donovani* prevented disruption of mitochondrial membrane potential. Furthermore, menadione or miltefosine treatment in miltefosine-unresponsive *L. donovani* had no effect on mitochondrial membrane potential (Figure 2.8).

***2.4.5 Intracellular Ca^{2+} measurement*:** The increase in cytosolic Ca^{2+} concentration in miltefosine-responsive and miltefosine unresponsive *L. donovani* promastigotes either treated or untreated with IC_{50} doses of miltefosine or menadione for 6 hours are shown in Figure 2.9. As shown, the measured fluorescence intensity at 510 nm in the miltefosine-responsive cells pre-treated with IC_{50} doses of miltefosine or menadione increased, indicating the formation of a larger amount of Ca^{2+}-MagFura complex. These data indicate disruption of mitochondrial membrane potential. Pre-treatment with NAC, a scavenger of ROS, before miltefosine or menadione treatment led to a cytosolic Ca^{2+} concentration similar to that in controls. Overall, the results suggest that the increase in Ca^{2+} concentration is linked to ROS accumulation. It is worth mentioning that miltefosine-unresponsive *L. donovani* showed less ROS accumulation and Ca^{2+} release than miltefosine-responsive *L. donovani*, and no mitochondrial membrane damage.

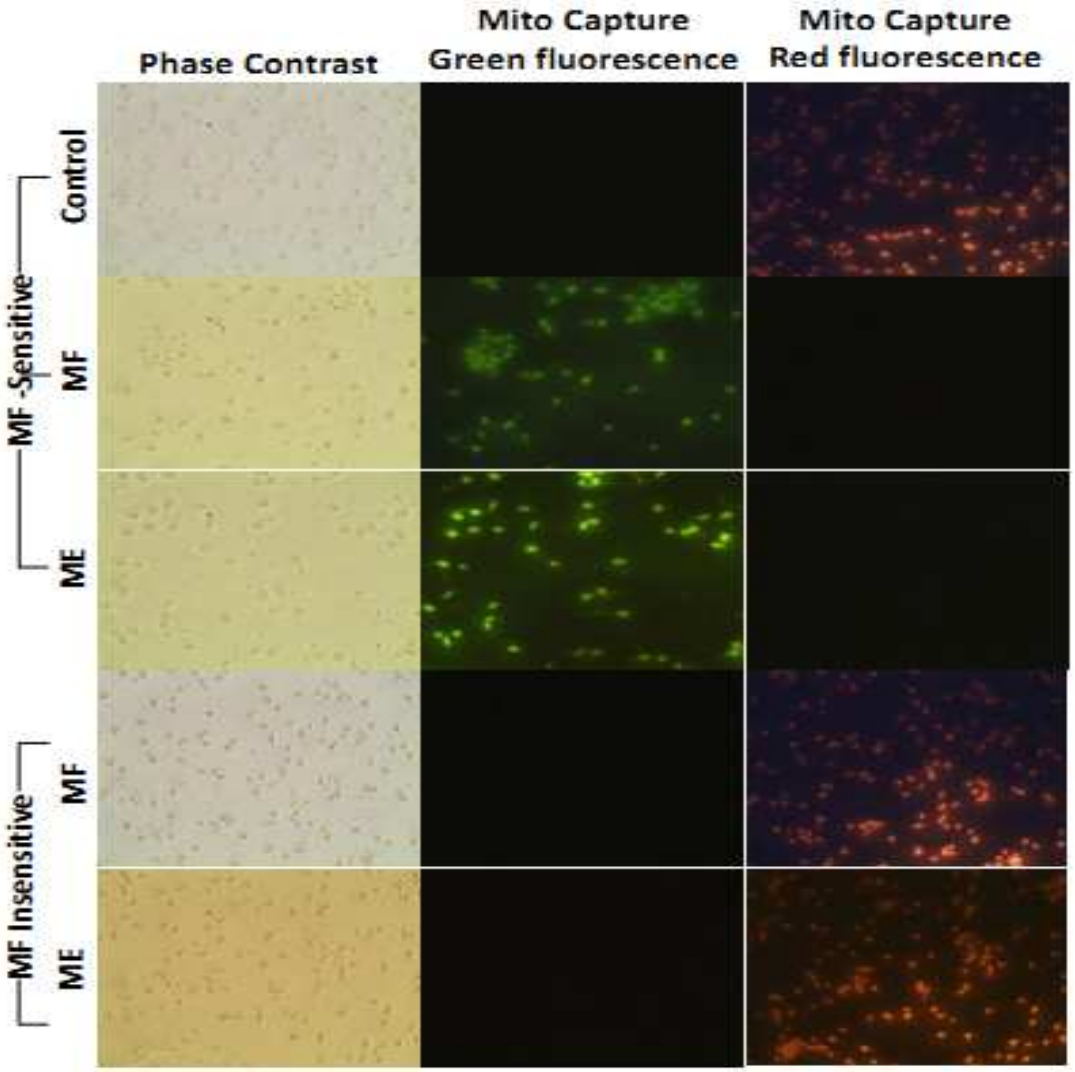

Figure 2.8: Miltefosine and menadione induced apoptosis as measured by mitochondrial membrane injury. *L. donovani* promastigotes were treated separately with IC50 doses of miltefosine and menadione for 6 h, and stained with a mitochondria-specific cationic dye, MitoCapture (Calbiochem). Untreated *L. donovani* promastigotes were used as controls. MF and ME represent miltefosine-treated and menadione-treated *L. donovani* promastigotes, respectively. Control, miltefosine-treated and menadione-treated cells were photographed with excitation wavelengths of ~ 570 nm (red) and ~ 500 nm (green), respectively, under a fluorescence microscope (X60). Normal mitochondrial membrane potential is indicated by red fluorescence, and depolarization of the mitochondrial membrane is indicated by green fluorescence. The data shown are representative of at least three experiments that gave similar results.

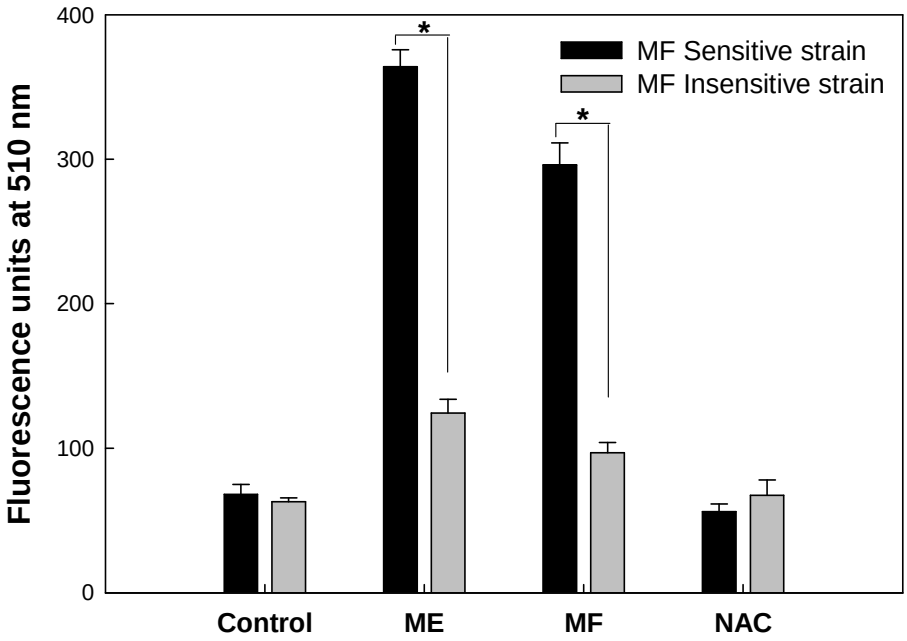

Figure 2.9: Increase in cytosolic Ca^{2+} concentration in miltefosine responsive and miltefosine-unresponsive *L. donovani* promastigotes separately treated with IC_{50} dose of miltefosine (MF) or menadione (ME) for 6 h and stained with Fura2AM. Control data indicates *L. donovani* promastigotes without any treatment; NAC data indicate NAC pre-treatment before miltefosine or menadione treatment. Fluorescence was measured with excitation and emission at 340 nm and 510 nm, respectively. Data represent the mean ± standard deviation of three independent experiments. Data with *P* values of <0.05 (*) were considered statistically significant.

***2.4.6 Redox enzyme assay*:** Miltefosine-unresponsive *L. donovani* showed higher superoxide dismutase (SOD) activity (466 unit mg protein^{-1}) and ascorbate peroxidase (APX) activity (2.6 mM min^{-1} mg protein^{-1}) than miltefosine-responsive *L. donovani* (400 unit mg protein^{-1} and 1.5 mM min^{-1} mg protein^{-1}, respectively) (Figure 2.10). The increase in activity of redox enzymes in miltefosine unresponsive *L. donovani* confirms that the mechanism of resistance against miltefosine is multifactorial, and that redox metabolism plays an important role. The data suggest that integrating redox modulators into conventional chemotherapy against leishmaniasis may be a viable strategy against miltefosine-resistance *Leishmania* isolates.

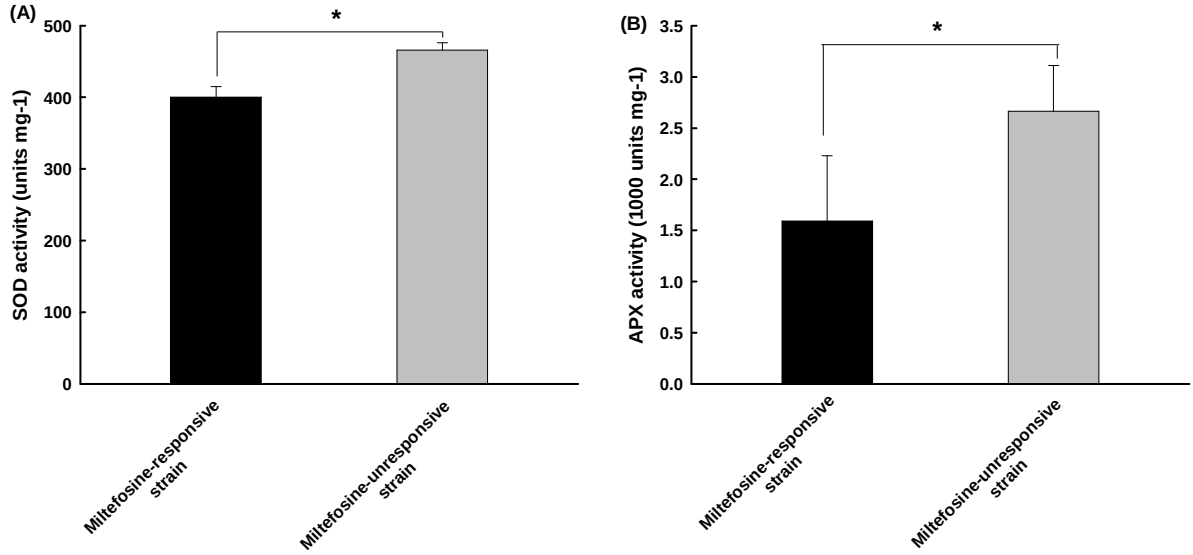

Figure 2.10: Superoxide dismutase and ascorbate peroxidase assay: Miltefosine unresponsive *L. donovani* showed higher superoxide dismutase and ascorbate peroxidase assay activity than miltefosine responsive *L. donovani*. Data represents the mean ± standard deviation of three independent experiments. Data with *P* values of <0.05 (*) were considered statistically significant.

2.5 Conclusion

Several studies on miltefosine-unresponsive *Leishmania* have been performed (*Pérez-Victoria et al., 2006; Seifert et al., 2007; Rakotomanga et al., 2005*). All studies support a role of fatty acid and steroid metabolism, as well as role of mutations in the miltefosine transporter *Ld*MT. Although there has been a substantial increase in our knowledge relating to miltefosine resistance in *Leishmania*, there is still much that is not understood. The role of ROS in killing *Leishmania* parasites is well documented (*Horta et al., 2012; Shukla et al., 2011; Shukla et al., 2012*). For the first time, we report experimental data that explain the relationship between parasite defence against reactive oxygen species and *Leishmania* resistance to miltefosine. Our microarray data suggest that the metabolism of miltefosine-unresponsive *Leishmania* is better at resisting oxidative stress. The biochemical studies reported in the present article conclusively shows that miltefosine-responsive *Leishmania* and miltefosine-unresponsive *Leishmania* promastigotes have different abilities to resist reactive

oxygen species. Our data indicate that miltefosine-unresponsive *Leishmania* accumulates a lower number of reactive oxygen species under conditions that are sufficient to cause reactive oxygen species-induced mitochondrial membrane potential damage and apoptotic death of miltefosine responsive *Leishmania*. Additionally, we found higher superoxide dismutase and ascorbate peroxidase activity in miltefosine-unresponsive *Leishmania*. Superoxide dismutase is a vital enzyme that detoxifies superoxide to hydrogen peroxide, H_2O_2 (less toxic than superoxide) and oxygen, and ascorbate peroxidase scavenges H_2O_2 and converts it into water. Superoxide dismutase and ascorbate peroxidase protect the parasite from oxidative stress and subsequent damage. There is a direct correlation between reactive oxygen species and mitochondrial damage and change in mitochondrial membrane potential. The results conclusively show that miltefosine-unresponsive *Leishmania* has an improved ability to resist reactive oxygen species. Miltefosine and many other drugs against *Leishmania* cause redox imbalance in the parasite. The widely used antimony-based drugs have also been reported to act via important enzymes involved in redox homeostasis (*Baiocco et al., 2009*). The mechanism of miltefosine resistance in *Leishmania* mediated through the redox system seems to be an adaptive change in the parasites resulting from the indiscriminate use of drugs.

Miltefosine and many other drugs against *Leishmania* cause redox imbalance in the parasite. The widely used antimony-based drugs have also been reported to act via important enzymes involved in redox homeostasis (*Baiocco et al., 2009*). The mechanism of miltefosine resistance in *Leishmania* mediated through the redox system seems to be an adaptive change in the parasites resulting from the indiscriminate use of drugs.

CHAPTER III

Ornithine decarboxylase of *Leishmania donovani:* Biochemical properties and possible role of N-terminal extension.*

3.1 Abstract

Leishmaniasis is a wide spread tropical disease caused by protozoan parasite *Leishmania* which belongs to order kinetoplastida and family trypanosomatidae. Ornithine decarboxylase is a key enzyme in polyamine biosynthesis in *Leishmania donovani*. Here, we report biochemical characterization of ornithine decarboxylase from *L. donovani*. Furthermore, we have also investigated the role of N-terminal extension (250 amino acids) found in ornithine decarboxylase of *L. donovani (Ld*ODC). The removal of N-terminal extended region of *Ld*ODC results in improved stability of the protein. However, the truncated *Ld*ODC does not show any activity. Apparently, while N-terminal extended region of *Ld*ODC helps in proper folding of the protein for catalytic activity, there is a stability trade-off. The native full length *Ld*ODC with N-terminal extension has activity but lower stability. Thus, there is trade off of conformational stability for enzyme activity. Comparison of biochemical properties of both, full-length and truncated enzymes, have provided interesting insights about the role of N-terminal extension in the protein.

Keywords: Ornithine decarboxylase; Leishmaniasis; N-terminal extension

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3.2 Introduction

Ornithine decarboxylase (*Ld*ODC) is an important enzyme of redox metabolism. It catalyzes the decarboxylation of ornithine to putrescine with the help of a co-factor pyridoxal 5'-phosphate (*Krauth-Siegel et al., 2008*). Each monomer of the enzyme has two domains, α/β -barrel domains and β-sandwich domain (*Stuart et al., 2008; Almrud et al., 2000; Kidron et al., 2007*).

*Ld*ODC (EC 4.1.1.17) is reported to be indispensable for survival of *L. donovani*, the causative organism of visceral leishmaniasis, and several other organisms (*Boitz et al., 2009; Jiang et al., 1999*). However, in some bacteria, plants and invertebrates; alternate pathways of putrescine biosynthesis have been reported (*Regunathan et al., 2000*). In our previous studies, we have found that *L. donovani* is very sensitive to imbalance in redox metabolism (*Shukla et al., 2011; Saudagar et al., 2013*). As *Ld*ODC is vital for survival of parasite, we are trying to explore it as a possible drug target (*Boitz et al., 2009; Jiang et al., 1999*). To complete its life cycle, *Leishmania* is exposed to diverse environmental conditions where parasite resides ranging from warm blooded acidic macrophage to alkaline midgut of cold blooded sand flies. Considering these various conditions in the life cycle of parasite, any drug target enzyme needs to be well characterized.

From the multiple sequence alignment result, *Ld*ODC showed a weak PEST sequence (SEEEGETSLSGP) near C-terminus (Figure 3.1) (*Hanson et al., 1992*). The PEST sequences are signal sequences which contain proline, glutamic acid, serine and threonine and make the associated protein susceptible to degradation (*Rogers et al., 1986; Ghoda et al., 1990*). Due to weak PEST sequence in *Ld*ODC, the protein is at low risk of degradation. Human ODC contains two PEST sequences spanning from 293-333 and 423-449, making it susceptible to degradation with a short half-life (*Tamori et al., 1995*; *Svensson et al., 1997*). It has been reported that *Ld*ODC shares a unique characteristic with ˜ 250 amino acid long N-terminal extension shown in figure 3.1 (*Hanson et al., 1992*). As human ODC without this N-terminal extension remains active and natively folded, role of this N-terminal extension in *Leishmania donovani* remains unclear. A prior report about cloning and structural studies of

full length *Ld*ODC in PET-30 Xa/LIC vector is published (*Dufe et al., 2007*). In the earlier publication, a different expression host and assay method was used (*Dufe et al., 2007*). However, not much information about biochemical properties like pH and temperature optima as well as role of N-terminal extension was given in the publication. Here, we report the cloning, expression and purification of full length *Ld*ODC as well as truncated *Ld*ODC in pET-28a(+) vector. More interestingly, we have also explored the biochemical properties and possible role of N-terminal extensions of *Ld*ODC.

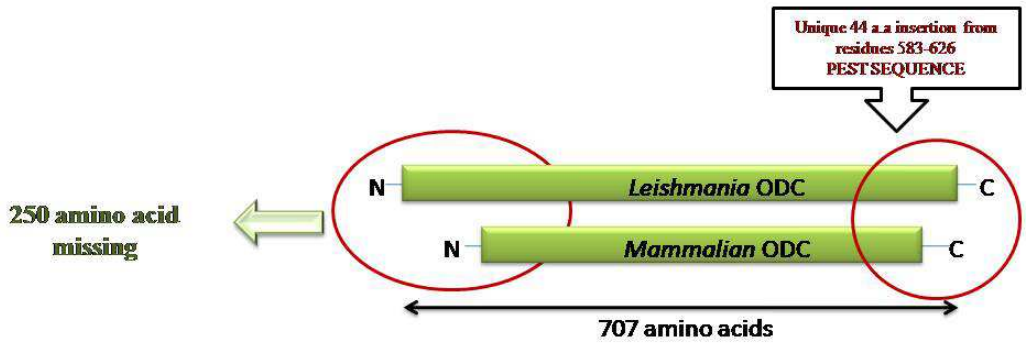


Figure 3.1: *Leishmania donovani* ornithine decarboxylase has extra N-terminal extension containing 250 amino acids compared to human ornithine decarboxylase. At the C-terminal, there is a weak PEST sequence.

3.3 Materials required and Methods

***3.3.1 Materials*:** Gene specific primers of *Ld*ODC, PCR clean up kits and Gel extraction kits were purchased from Qiagen, USA. T4 DNA ligase, *Nhe*1 and *Xho*1 were procured form New England Biolabs, USA. Plasmid isolation kit, DMSO, PEG, Kanamycin, Isopropyl β-D-thiogalactopyranoside (IPTG), Tris-HCl, Dithiotritol (DTT), Ethylenediamine-tetra acetic acid (EDTA), Phenylmethane sulphonyl fluride (PMSF), β-mercapto ethanol, Sodium dodecyl sulphate (SDS), Lysozyme and Pyridoxal phosphate (PLP) were purchased from Sigma-Aldrich, USA. All other chemicals used in the experiments were of the highest grade procured from Sigma-Aldrich, USA and Merck, USA. *Ld*ODC gene in pSNBR vector was gifted by Dr. S.C. Robert, School of Pharmacy at Pacific University, USA. The details of pSNBR vector are already published (*Jiang et al., 1999; Hanson et al., 1992*). *Ld*ODC

cloned in pSNBR vector was used as sources for amplification of full-length *Ld*ODC and truncated *Ld*ODC (T-*Ld*ODC) genes. The amplified gene was further cloned in pET-28a(+) vector for expression in *E.coli*.

***3.3.2 Cloning of both full length and truncated ornithine decarboxylase in* pET-28a(+) *vector*:** *Ld*ODC gene in pSNBR vector was used as a template for the amplification of full-length *Ld*ODC and truncated *Ld*ODC (T-*Ld*ODC) gene. The coding region of full length *Ld*ODC was amplified by polymerase chain reaction (PCR) using the: forward primer (5'-GATCGCTAGCATGGGTGATCATGACGT-3') and reverse primer (5'-GGAATTCTCGAGTCACTCGCTCACACACCT-3') containing restriction sites for *Nhe*I and *Xho*I respectively. For T-*Ld*ODC, PCR amplification was done using: forward primer (5'-GATCGCTAGCGAGGATCCATTCTACATCATCG-3') and reverse primer (5'-GGAATTCTCGAGTCACTCGCTCACACACCT-3'), also containing restriction sites for *Nhe*I and *Xho*I, respectively. The conditions used for PCR were as follows: initial denaturation 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 40 s, extension at 72°C for 2 min and final extension at 72°C for 10 min. The purified PCR fragments were digested with *Nhe*I and *Xho*I restriction endonucleases and cloned into the *Nhe*I and *Xho*I sites of the pET-28a(+) vectors which had been digested with the same enzymes, generating fusion expression vectors pET28a-*Ld*ODC and pET28a-T-*Ld*ODC. The resultant plasmids (expression constructs) were transformed into *E.coli* DH5α competent cells by heat shock method to increase the copy number of plasmid. Transformed colonies were identified by PCR amplification using gene specific primer and double digestion of plasmid by restriction endonucleases (*Nhe*I and *Xho*I) and by sequencing (Figure 3.4). Detail information of *Ld*ODC and T-*Ld*ODC is given in Table 3.1.

Table 3.1: Detail information about *Ld*ODC and T-*Ld*ODC

	Full length ODC	**Truncated ODC**
Isoelectric point	5.49	4.95
DNA size	2121 bp	1371 bp
No. of amino acids	707	457
Molecular weight	77.4 kDa	50.2 kDa

***3.3.3 Sequence Analysis*:** Ornithine decarboxylase of various species was retrieved from the NCBI database. Blast hits were aligned using CLUSTAL W and alignments were drawn using ESpript 3.0. Results of sequence alignment show a weak PEST sequences near C-terminus amino acid 583 to 626 (Figure 3.3).

***3.3.4 Expression and purification of both full length and truncated ornithine decarboxylase in E.coli BL21 (DE3)*:** Confirmed pET28a-*Ld*ODC and pET28a-T-*Ld*ODC constructs were further transformed into the BL21 (DE3) *E.coli* expression cells by heat shock method. Single colony of BL21(DE3) expression cells harboring expression construct pET28a-*Ld*ODC and pET28a-T-*Ld*ODC, were inoculated into 4 ml Luria Bertani (LB) media supplemented with kanamycin (50µg/ml) and grown at 37°C, 180 rpm overnight. The overnight grown culture was then diluted to 1:100 with LB media containing 50µg/ml kanamycin and grown at 37°C, 180 rpm until the OD_{600} reached at 0.45-0.5. The bacterial cells were induced with 0.5 mM IPTG and growth was continued at 18°C, 140 rpm for 6 h. The induced culture was harvested by centrifugation at 7500 rpm for 5 min at 4°C and stored at -80°C till purification. The cell pellet was re-suspended into ice-chilled 8ml lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 2.5% glycerol, Lysozyme 0.7 mg/ml, and 0.2 mM PMSF) and subjected to sonication on ice with a pulse of 5 s on and 9 s off for 6 min (*Dufe et al., 2007; Preeti et al., 2012*). The obtained cell lysate was centrifuged at 12,000 rpm for 20 min at 4°C and supernatant was collected. The supernatant obtained was applied to Nickel-affinity column pre-equilibrated with 5 column volumes of 50 mM Tris-HCl, 250 mM NaCl and 10 mM imidazole pH 7.5. Then column was washed with same buffer to remove non-specifically bound contaminants. Finally, bound protein was eluted in same buffer containing 250mM imidazole and dialyzed against 20 mM sodium phosphate, 250 mM NaCl, 2.5% glycerol, pH 7.5 to remove imidazole. During process of optimization, 250 mM NaCl was found to be essential for keeping protein in solution. Eluted fractions were analyzed on SDS-PAGE.

***3.3.5 Activity assay of ornithine decarboxylase*:** Activity of ODC was spectrophotometrically determined by a method described earlier (*Badolo et al., 1999*). This

method was used earlier for ODC of other organism but for the first time we have used this method in case of *Leishmania* ODC. Our current method is based on the reaction between SAO (soyabean amine oxidase) and putrescine, the product of ornithine decarboxylase catalyzed reaction (Figure 3.2). Crude soyabean amine oxidase enzyme was used from soyabean plant. Equal amount of plant used to blend in equal volume of buffer. Plant section used is whole seedling excluding seed (young plant raised from seven day long germinated soyabean seed). The plantlets were mechanized in ice-chilled 50 mM Tris-HCl buffer, pH 8.0, centrifuged at 12,000 rpm for 15 min and then supernatant was used as crude SOA enzyme in the reaction. The buffer of purified ODC protein was exchanged with 20 mM sodium phosphate, 250 mM NaCl and 0.2 mM DTT buffer (pH 7.5). The reaction mixture contained 0.1 mM EDTA, 0.1 mM PLP, 1 mM of L-ornithine and 10 µl (3 µg) of protein to which 20mM sodium phosphate and 250 mM NaCl buffer (pH 7.5) was added to make the final volume to 50 µL. The reaction mixture was incubated at 37°C for 90 minutes. Further, with the above reaction 25 µl of SAO, 100 µg/ml phenol, 100 µg/ml 4-aminoantipyrine and 18 µl horseradish peroxidase was added making final volume to 250 µl by adding 50 mM Tris-HCl, 250 mM NaCl pH 7.5. The reaction was incubated at 25 °C for 60 min. The concentration of putrescine formed by *Ld*ODC catalysis was calculated by measuring the absorbance of the coloured complex (505 nm) formed as a result of the reaction of H_2O_2 with 4-aminoantipyrine and phenol catalyzed by horseradish peroxidase. For negative control, purified protein or substrate was replaced with buffer in the *Ld*ODC enzyme reaction mixture.

***3.3.6 Estimation of pH and temperature optima of LdODC*:** Estimation of temperature optima of *Ld*ODC was done in presence of different buffers of pH range (01 to 12) namely 50 mM KCl-HCl (pH-1.0-1.5), 50 mM glycine-HCl (pH-2.0-3.5), 50 mM sodium acetate (pH 4.0-5.5), 50 mM sodium phosphate buffer (pH 6.0-7.5), 50 mM Tris-HCl (pH 8.0-10.5), 50 mM $NaHCO_3$-NaOH (pH-11) and 50 mM KCl-NaOH (pH-12) (*Saudagar et al., 2011; Singh et al., 2011*). The substrate was also prepared in the respective buffers and incubated with the enzyme (1 mg) at room temperature (25°C) for enzymatic assay. At each pH, a control assay was carried out without the enzyme and was used as a blank.

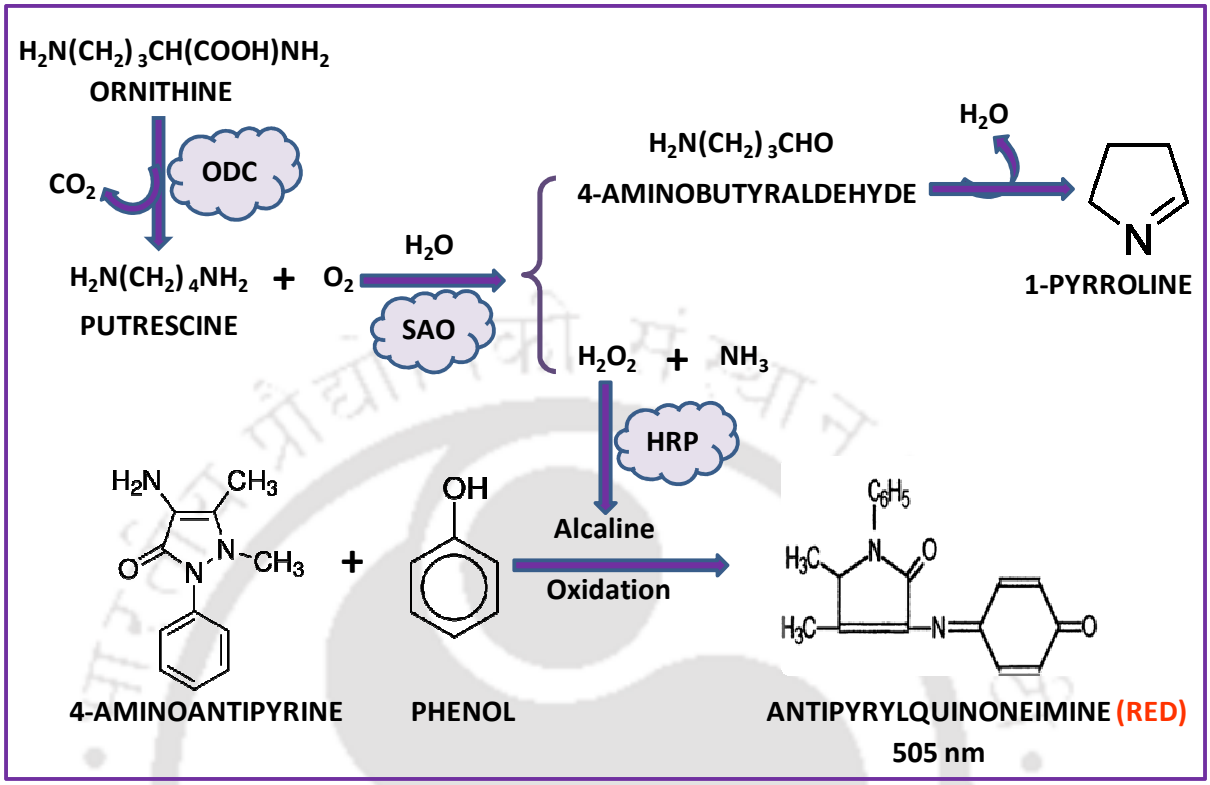


Figure 3.2: Schematic diagram of ornithine decarboxylase activity assay: Ornithine decarboxylase (ODC) converts ornithine to putrescine. Putrescine cannot be spectrophotometrically determined. Therefore, this reaction is coupled with soyabean amine oxidase (SAO), which converts putrescine into hydrogen peroxide and 4-aminobutryaldehyde. This reaction is further coupled with horseradish peroxidase (HRP) enzyme and to this reaction phenol and 4-aminoantipyrin is also added. HRP oxidises phenol and 4-aminoantipyrin in presence of H_2O_2 and give rise to antipyrlquinoneimine which is spectrophotometrically determined at 505 nm.

Similarly activity assay for *Ld*ODC was carried out at different temperatures, ranging from 10 to 100 °C. Prior to the assays, substrate solution was also equilibrated at the corresponding temperature in the same buffer. At each temperature, a control assay was carried out without the enzyme and was used as a blank.

***3.3.7 Spectroscopic measurements*:** Circular dichroism (CD) measurement in far UV range was performed using Jasco J715 spectropolarimeter equipped with constant temperature cell holder. Circular dichroism spectra was collected in the range of 190 to 250 nm using a quartz

cuvette of 1 mm path length and scan speed of 20 nm/min. Final protein of 5 μM was in 10 mM sodium phosphate buffer pH 7.5 (*Shukla et al., 2011*)

Fluorescence measurements were taken using Varian Fluorescence Spectrofluorometer. The sample was excited at 295 nm and emission scan was collected from 300 nm to 400 nm. Slit widths for both excitation and emission were kept at 5 nm. Final protein concentration for fluorescence measurements was 1 μM in 20 mM sodium phosphate buffer pH 7.5. GuHCl (6 M) was used as a denaturing agent. Samples were incubated overnight with denaturant before taking spectra.

***3.3.8 Guanidine hydrochloride (GuHCl) induced unfolding*:** GuHCl induced denaturation was performed at neutral pH (20 mM phosphate buffer pH 7.5) with increasing concentrations of GuHCl. Protein sample (1 μM) was incubated at a desired denaturant concentration for overnight at 25^{o}C to attain equilibrium. Unfolding of the protein was measured by fluorescence spectroscopy. Data was converted into fraction unfolded F_u using the following equation:

$$F_u = (F_{obs}-F_n) / (F_u-F_n)$$

where, F_{obs} is the observed value of the signal concentration of GuHCl, F_n and F_u are the values of native and unfolded protein respectively (*Khan et al., 2007; Jana et al., 2006*)

3.4 Results and discussion

***3.4.1 Sequence Analysis*:** Multiple sequence alignment of ornithine decarboxylase was done ClustalW and shown (Figure 3.3) using ESPript (http://espript.ibcp.fr/ESPript/ESPript/) .

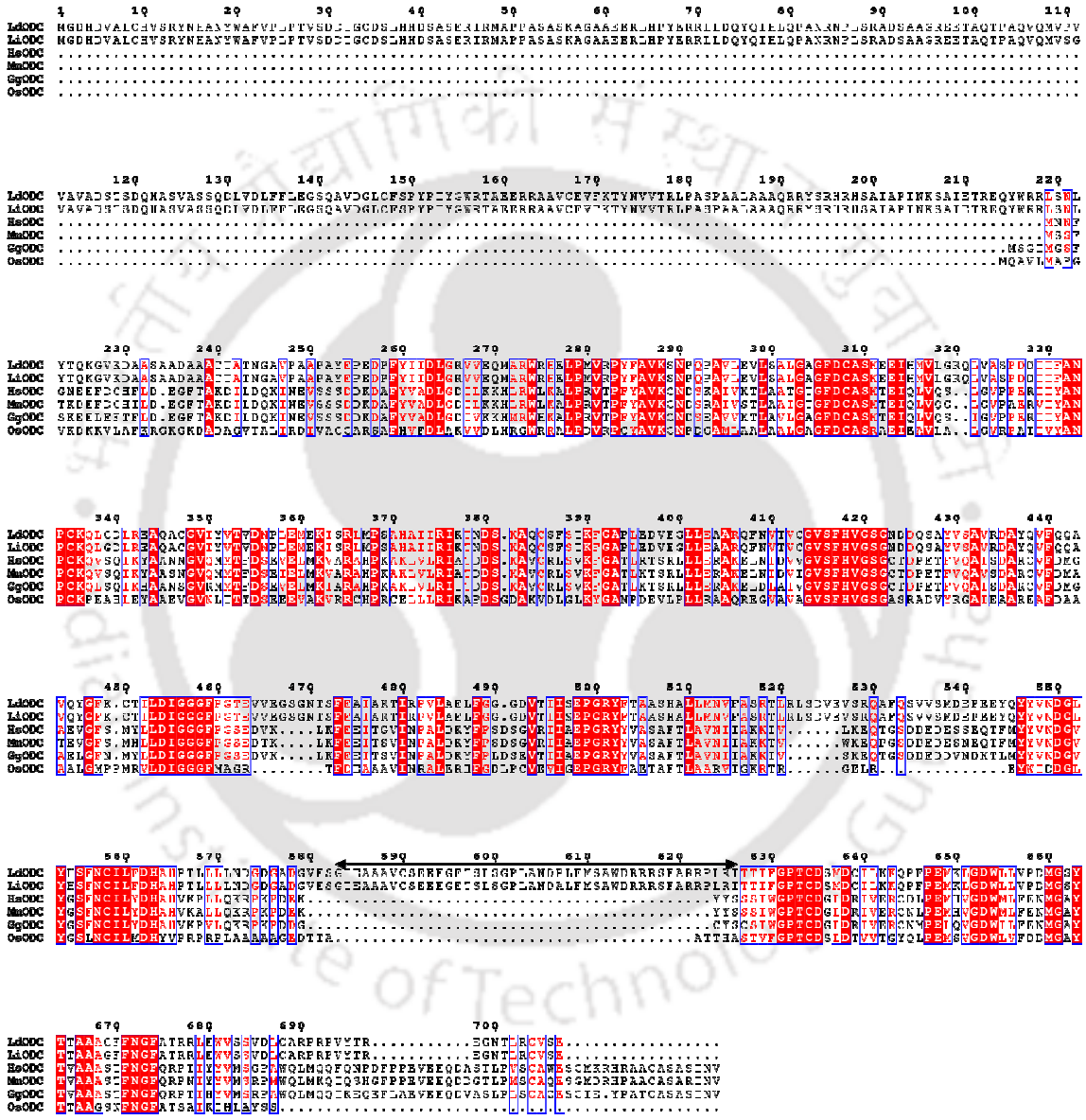


Figure 3.3: Multiple sequence alignment of ornithine decarboxylase (ODC) of different organism. *Ld*ODC: *Leishmania donovani* ODC (AAA29259.1), *Li*ODC: *Leishmania infantum* ODC (XP_003392267.1), *Hs*ODC: *Homo sapiens* ODC (NP_002530.1), *Ms*ODC: *Mus musculus* ODC (NP_038642.2), *Gg*ODC: *Gallus gallus* ODC (NP_001161238.1), *Os*ODC: *Oryza sativa* ODC (NP_001063827.2). Weak PEST sequence in *Ld*ODC is shown by an arrow.

We found that N-terminal extension is missing in *Homo sapiens* (human), *Mus musculus* (house mouse), *Gallus gallus* (Red junglefowl), *Oryza sativa* (rice). Extra sequence at N-terminal (approx. 250 amino acids) present in *L. donovani and L. infantum*.

***3.4.2 Cloning of both full length and truncated ornithine decarboxylase in* pET-28a(+) *vector*:** A putative *L. donovani* ODC gene predicted to encode a protein of 707 amino acids was amplified from ODC containing pSNBR vector (Figure 3.4). The authenticity of the gene was initially confirmed by doing PCR of ODC containing pSNBR vector showing an intense band size of 2121 bps (Figure 3.4A). Amplified *Ld*ODC gene was then cloned into pET28a(+) vector isolated from *E.coli* (Figure 3.4B).

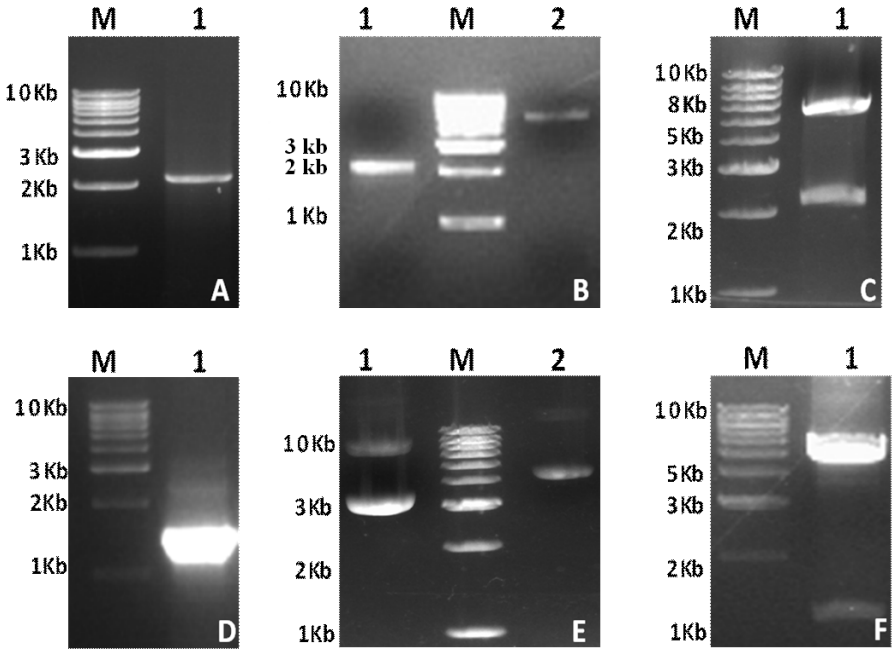


Figure 3.4: Cloning of *Ld*ODC **(A)** M: 1 kb ladder, Lane 1: PCR amplification of *Ld*ODC gene, **(B)** Lane 1: PCR amplification of *Ld*ODC, M: 1 kb DNA ladder, Lane 2: plasmid isolation of pET28a(+) vector, **(C)** M: 1kb DNA ladder, Lane 1: confirmation of positive clone by double digestion (*Nhe*1 and *Xho*1) of recombinant vector pET28a-*Ld*ODC**,** showing release of *Ld*ODC gene fragment, **(D)** M: 1kb DNA ladder, Lane 1: PCR product of T-*Ld*ODC, **(E)** Lane1: T-*Ld*ODC in TA vector, M: 1 kb DNA ladder, Lane 2: plasmid isolation of pET28a(+) vector, **(F)** M: 1 kb DNA ladder, Lane 1: showing release of insert after double digestion by *Nhe*I/*Xho*I of recombinant vector pET28a-T-*Ld*ODC.

Positive clones were screened by doing PCR which showed band at the same size (2121 bp), and the clone was further confirmed by restriction digestion with *Nhe*I *and Xho*I restriction enzyme, which cuts the vector pET28a(+)-*Ld*ODC and releases back the insert, i.e. products of ~2.1 kbp and ~6.3 kbp fragments (Figure 3.4C). Similarly, amplification of T-*Ld*ODC of size 1371 bp has been done using ODC containing pSNBR vector (Figure 3.4D). Double digestion of positive clone for T-*Ld*ODC showed release of insert (Figure 3.4F). Finally two individual sequencing results confirmed *Ld*ODC and T-*Ld*ODC clones.

3.4.3 Expression and purification of both full length and truncated ornithine decarboxylase in E.coli BL21 (DE3): Both recombinant constructs were transformed into BL21 (DE3) *E.coli* expression cells separately. In order to optimize the expression of recombinant proteins, the transformed culture was grown at different temperatures ranging from 18 °C to 25 °C, but most of the recombinant protein was found in pellet. Very small amount of protein was found in soluble fractions after *E.coli* cells lysis and centrifugation. We have grown culture in large volume in order to get more protein. The soluble fraction of *Ld*ODC or T- *Ld*ODC was purified with typical yield of ~1 mg/L. Although, the yield was less, the amount was sufficient for biochemical studies. The purity of the protein was judged using SDS-PAGE (Figure 3.5). The purified protein was used for studies within 1-2 weeks. The protein loses its activity after prolong storage.

***3.4.4 Biochemical characterization of ornithine decarboxylase*:** The activity assay method is a coupled enzyme reaction, since we cannot directly estimate formation of putrescine, therefore we have employed putrescine standard curve (Figure 3.6A). From this standard curve, amount of putrescine formed in presence of different concentrations of ornithine (substrate of first coupled reaction) was estimated by correlating the absorbance value. We considered this value as v after dividing it by 90 (total reaction time is 90 min) and graph was plotted. Kinetic parameters like K_m and V_{max} of purified *Ld*ODC were estimated by Line-weaver Burk plot (Figure 3.6B). K_m value of *Ld*ODC for ornithine (substrate) was calculated to be 0.418 ± 0.05 mM and V_{max} was 10.71 µmoles/min.

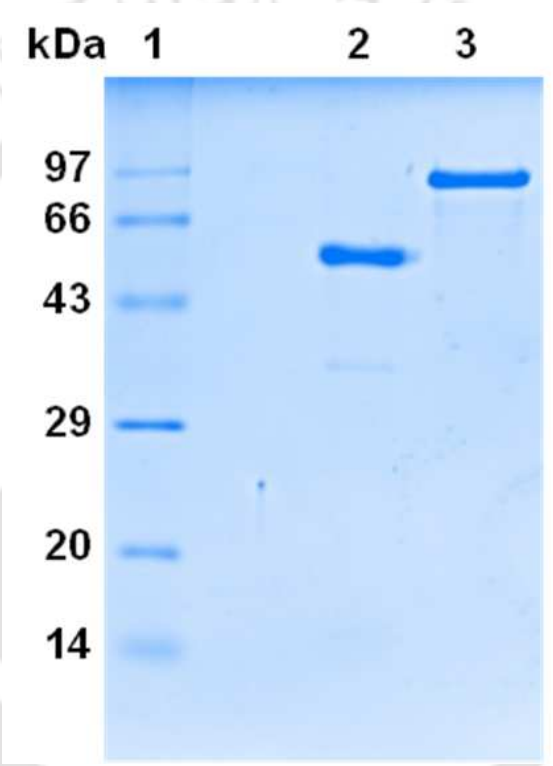


Figure 3.5: SDS-PAGE image of purification of both full length and truncated ornithine decarboxylase. Lane 1: protein molecular weight marker, lane 2: purified T-*Ld*ODC, lane 3: purified *Ld*ODC.

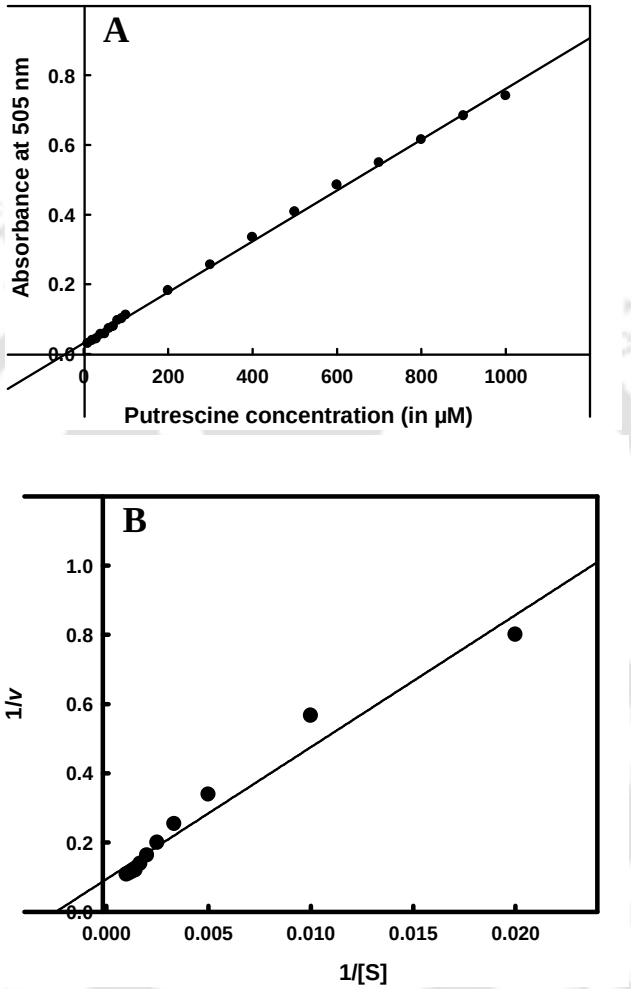


Figure 3.6: (A) Putrescine standard curve, **(B)** Line-weaver Burk plot of *Ld*ODC enzyme assay in varying concentrations of ornithine as a substrate. Calculated K_m is 0.409 mM and V_{max} is 10.71 µmoles per min per mg protein.

***3.4.5 pH and temperature optima of LdODC*:** A mixed buffer system was used to determine the optimum pH range for *Ld*ODC (Figure 3.7A). The enzyme remains active in pH range from 6-10, but optimum activity is found at pH 7.5. Activity assay was performed at different temperatures for *Ld*ODC. It has an optimum activity at 37 °C, but remains in active form in a range of 10 to 50 °C. The activity gradually decreases along with increase in above optimum temperature (Figure 3.7B).

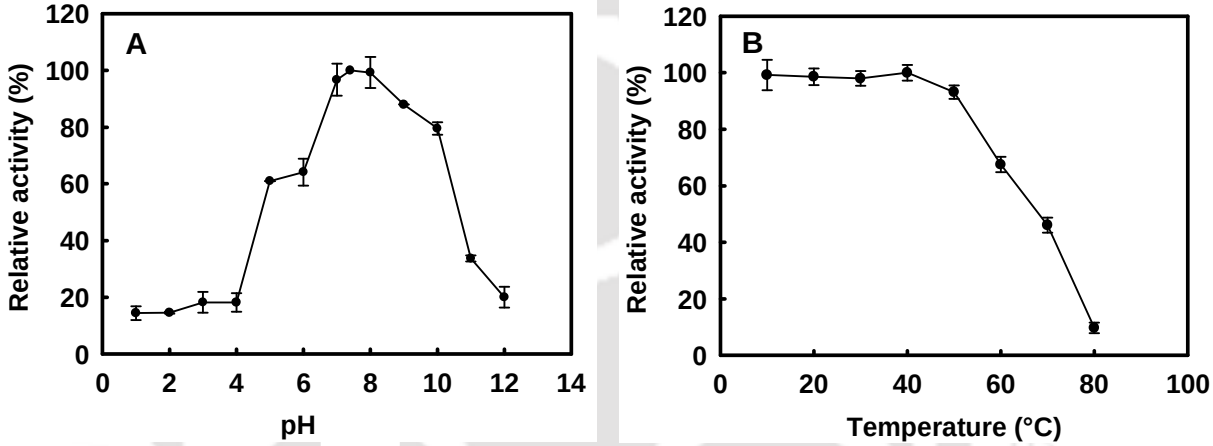


Figure 3.7: Enzymatic properties of ornithine decarboxylase. **(A)** pH optima for *Ld*ODC: activity assay was carried out in different buffer systems having different pH, and optimum pH is found to be 7.5, **(B)** Temperature optima for *Ld*ODC: activity assay carried out in different temperature, and optimum temperature is 37°C.

***3.4.6 Spectroscopic studies of both full length and truncated ornithine decarboxylase*:** Secondary structure of the two forms of enzyme was studied by fluorescence spectra in native and 6 M GuHCl containing buffer. The data suggests a change in the tryptophan environment of T-*Ld*ODC as evident from large decrease in fluorescence intensity and minor change in wavelength maxima (Figure 3.8A). The denatured proteins (*Ld*ODC and T-*Ld*ODC) showed large red shift in wavelength maxima suggesting exposure of tryptophan. Moreover T-*Ld*ODC showed more ordered secondary structure as evident from increasing far

UV CD ellipticity (Figure 3.8B). Overall, the data suggested that there was a structural change after truncation of N-terminal extension of *Ld*ODC.

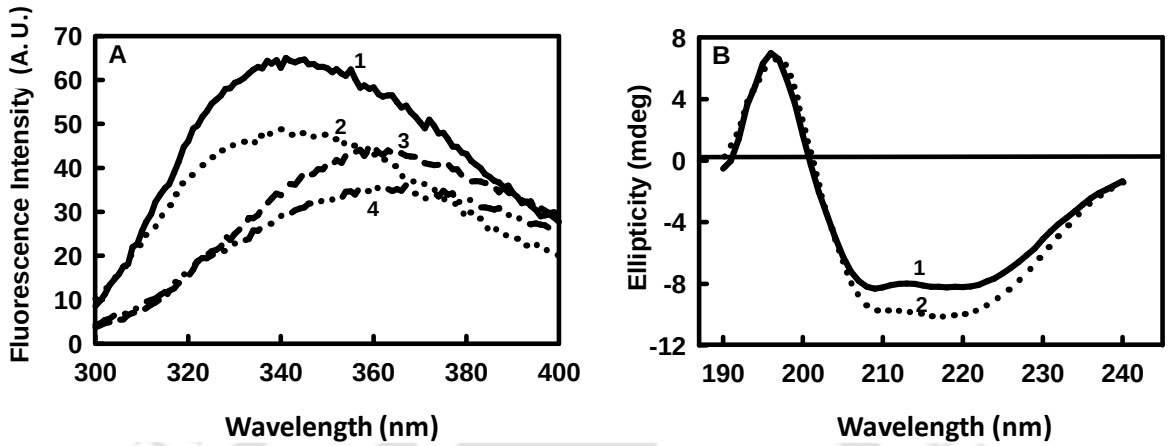


Figure 3.8: Spectroscopic properties of *Ld*ODC **(A)** Fluorescence spectra in 50 mM phosphate buffer 7.4 **(B)** Far UV CD spectra in 50 mM phosphate buffer 7.4 Spectra denoted by 1, 2, 3 and 4 in the figure represents data for full length *Ld*ODC, N-terminal extension truncated *Ld*ODC, length *Ld*ODC in 6 M GuHCl and N-terminal extension T-*Ld*ODC in 6 M GuHCl, respectively.

***3.4.7 Guanidine hydrochloride induced unfolding and stability comparison of full length and truncated ornithine decarboxylase*:** Analysis of guanidium hydrochloride (GuHCl) induced denaturation curves provides a measure of the conformational stability of the proteins. The data is plotted in terms of fraction unfolded for stability comparison of *Ld*ODC and T-*Ld*ODC (Figure 3.9). The T-*Ld*ODC shows transition midpoint of 2.3 M while *Ld*ODC has transition midpoint of 1.8 M. The data clearly indicates that the T-*Ld*ODC has higher stability compared to *Ld*ODC. The protein appears to have a balance between two characteristics, i.e. stability and activity. While removal of N-terminal extension leads to significant increase in the stability of the enzyme, activity of the enzyme is lost after truncation. In order to fold *Ld*ODC in an active conformation, N-terminal extension is required at the cost of stability. This is an excellent example of enzyme trading stability for function.

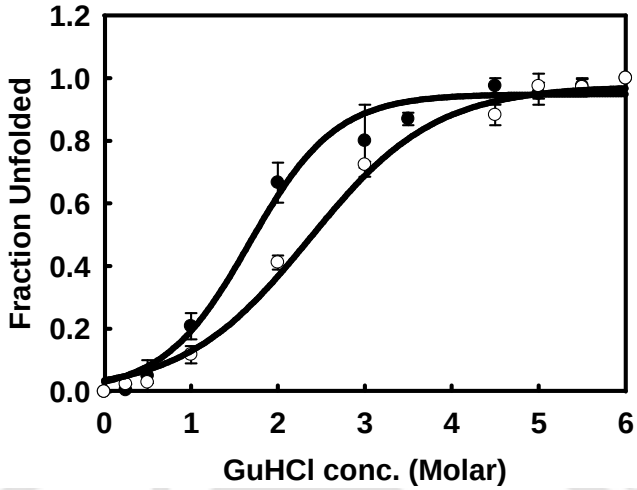


Figure 3.9: GuHCl induced equilibrium unfolding of (●) *Ld*ODC and (○) T-*Ld*ODC**:** GuHCl induced unfolding was carried out at pH 7.0. The unfolding transitions were followed by tryptophan fluorescence wavelength maxima after excitation at 295 nm. The data is plotted in terms of fraction unfolded for stability comparison of *Ld*ODC and T-*Ld*ODC. The data presented is an average of three independent experiments.

3.5 Conclusion

Ornithine decarboxylase is a drug target enzyme of *Leishmania donovani*. As a drug target, the enzyme needs to be well characterized for this purpose we have cloned and expressed *Ld*ODC and T-*Ld*ODC. Moreover, as the parasite resides in diverse conditions like acidic condition of macrophage cells of warm blooded mammals and alkaline midgut of sand fly, hence a detailed characterization of the enzymatic activity in various conditions was carried out. T-*Ld*ODC (*Ld*ODC without N-terminal extension) did not show any enzymatic activity suggesting the importance of N-terminal extension for either enzyme activity or proper folding leading to inactive enzyme. As active human ornithine decarboxylase does not have N-terminal extension, it is speculated that the N-terminal extension is necessary for proper folding of *Ld*ODC. The spectroscopic characterization suggested structural changes after removal of N-terminal extension. The stability characterization of *Ld*ODC and T-*Ld*ODC showed that the T-*Ld*ODC had higher stability. Taken together, the results point out towards

folding/activity and stability trade off. The N-terminal extension of *Ld*ODC helps in folding of the enzyme in active conformation at the cost of activity. In the experimental condition, when N-terminal extension in *Ld*ODC is removed (T-*Ld*ODC), the enzyme is more stable but cannot fold to its native conformation.

CHAPTER IV

Inhibition of ornithine decarboxylase of *Leishmania donovani* shows inhibitor specific effect: Mystery remains unsolved *

4.1 Abstract

Ornithine decarboxylase (*Ld*ODC), a key enzyme in polyamine biosynthesis in *Leishmania donovani*, catalyzes conversion of ornithine to putrescine that is finally used for synthesis of other polyamines. Inhibition of ornithine decarboxylase is likely to deplete the parasite trypanothione and may result in increased reactive oxygen species (ROS). Sequence as well as structure of *Ld*ODC and human ODC shows significant differences, therefore we have identified novel specific inhibitors of *Ld*ODC. These inhibitors are able to inhibit recombinant *Ld*ODC and decrease intracellular putrescine concentration showing target specificity. The *Ki* values of *Ld*ODC inhibition do not correlate with IC_{50} values in *Leishmania* promastigote. These inhibitors, except compound M-5, have only minor effect on *Leishmania* promastigotes and IC_{50} values are several fold higher compared to *Ki* values. In case of compound M-5, IC_{50} value is less than *Ki* value (the compound may target/affect other molecules also). Our studies suggest that the parasite resists these *Ld*ODC inhibitors by over-expression of spermidine synthase.

Keywords: Leishmaniasis; Putrescine; Inhibitors; Ornithine decarboxylase.

* Part of the work is communicated for publication

4.2 Introduction

Leishmaniasis is an existing neglected tropical disease, caused by over twenty different species of *Leishmania* parasite. *Leishmania* is a digenetic parasite which is transmitted from one host to other by the bite of female sandfly and manifests several clinical forms like visceral, cutaneous and mucocutaneous leishmaniasis (*Gradoni et al. 2008*). Visceral leishmaniasis, caused by *Leishmania donovani* in Indian subcontinent, is the most severe and fatal form of the disease (*Shukla et al. 2010*). The currently available treatments of the disease have severe limitations.

Polyamines play important role in the cellular homeostasis of most of the living organisms, including *Leishmania* parasite (*Coleman et al. 2004; Krauth-Siegel and Comini 2008*). Ornithine decarboxylase catalyzes the conversion of ornithine to putrescine, a precursor of other polyamines like trypanothione, a key redox metabolite in the parasite. Imbalance in the redox metabolism causes increase in reactive oxygen species (ROS). Our previous studies have shown the parasite to be highly sensitive to increase ROS (*Saudagar et al. 2013; Shukla et al. 2011; Saudagar et al. 2011; Shukla et al. 2012*). Ornithine decarboxylase (*Ld*ODC) of *Leishmania donovani* is reported to be essential for the parasite survival and infectivity. The study shows that the *Ld*ODC knockout strain was incapable of growing in polyamine-deficient medium (*Jiang et al. 1999; Boitz et al. 2009*). Moreover, the auxotrophy could be overcome by putrescine supplementation. Further, DL-α-difluoromethylornithine (DFMO), a non specific inhibitor of ornithine decarboxylase, is able to kill *Leishmania* promastigote (*Coons et al., 1990*). DFMO resistant strain of *Leishmania* survives in presence of DFMO, by a complex and partially deciphered mechanism involving many proteins (*Coons et al., 1990; Singh et al., 2014*). Some other inhibitors of *Ld*ODC have shown anti-leishmanial effects (*Hazra et al. 2013; Singh et al., 2014*). The current report shows a very different cellular effect of *Ld*ODC inhibition on *Leishmania* parasite. The parasite is able to survive the newly identified inhibitors by over-expressing spermidine synthase. It remains unclear, why parasite has inhibitor specific effect of *Ld*ODC inhibition.

4.3 Materials and Methods

***4.3.1 Parasites, cell lines and chemicals*:** The *Leishmania donovani* (MHOM/IN/2010/BHU1081) was obtained from Prof. Shyam Sundar, Banaras Hindu University, India and cultivated in M199 liquid media supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. The procedure is optimized and working in our laboratory (*Saudagar et al. 2013*; *Shukla et al. 2011*; *Saudagar et al. 2011*; *Shukla et al. 2012*).

H_2DCFDA dye was obtained from Invitrogen. Isopropyl β-D-thiogalactopyranoside (IPTG), Tris-HCl, dithiotritol (DTT), ethylenediamine-tetra acetic acid (EDTA), phenylmethane sulfonyl fluoride (PMSF), Lysozyme, pyridoxal-phosphate (PLP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) were obtained from Sigma. All other chemicals used in the experiments were of the highest grade procured from Sigma-Aldrich or Merck.

***4.3.2 In silico* identification of potential inhibitors for *Ld*ODC:** In our earlier report, we have done *in silico* docking studies with *Ld*ODC using a total of 35,889 compounds retrieved from ZINC database, which has 54,665 different stereo isomers (*Chakraborty et al. 2013*). The modelled structure of *Ld*ODC (PMBD ID: PM0077509) was used for the studies (*Chakraborty et al. 2013*). Since, few top hits of *in silico* predicted compounds did not show experimental inhibition on recombinant *Ld*ODC, top 100 hits of our previous docking studies were taken for further refinement using 100 LGA runs (ga_run 100), population size of 150 (ga_pop_size, 150) and number of energy evaluations of 175000 (ga_num_evals, 175000). All other docking parameters and procedure were used as reported earlier (*Chakraborty et al. 2013*). Docking simulation were performed using both *L. donovani* (*Ld*ODC) and human ornithine decarboxylase. The compounds showing best affinity towards *Ld*ODC and least affinity towards human ornithine decarboxylase was chosen as top hits with idea to identify inhibitor with higher preference for *Ld*ODC. The crystal structure of human ODC was retrieved from PDB (PDB ID: 2OO0). The ligands were sorted according to descending order of difference in free energy of binding, *i.e* the compound showing highest difference in free

energy of binding would be the topmost hit. Further, second criteria to choose best among them would be based on number of confirmations in largest cluster. Considering both the factors, compounds having highest difference in free energy of binding and highest number of confirmations in largest cluster would be placed at the top. Among them top seven were selected based on commercial availability. Similarly, mangiferin was also docked and analyzed. All selected compounds for biochemical studies based on *in silico* predictions are given in Table 4.1

***4.3.3 Inhibition study of Leishmania donovani ornithine decarboxylase (LdODC)*:** Dr. Robert, Pacific University School of Pharmacy has donated *Ld*ODC gene in pSNBR vector for the current work. The details of the vector with *Ld*ODC gene is already published (*Hanson et al. 1992*). The plasmid was used as a template for amplifying *Ld*ODC gene and cloned into pET-28a (+) vector. After confirmation of in-frame insertion of *Ld*ODC gene, the vector was transformed into BL21 (DE3) expression cells. His-tagged ODC was purified using nickel affinity chromatography. Details of cloning, expression and purification of the enzyme used for the study are already published (*Das et al. 2015, Hazra et al. 2013*). *Ld*ODC activity assay was performed using method described earlier, but first time used for *Leishmania* protein (*Badolo et al. 1999*). The *Ld*ODC was assayed for experimental inhibition with *in silico* identified hits. For inhibition studies, inhibitors were incubated with *Ld*ODC enzyme reaction assay mixture before coupling the reaction. Control experiments were done to confirm no effect of inhibitors on soyabean amine oxidase used for coupled enzyme assay. The *Ld*ODC inhibition studies were carried out using at a given concentration of inhibitor and varying concentration of substrate. A control experiment without any inhibitor was done for comparison and calculation of enzymatic parameters. The data is plotted in the form of Lineweaver-Burk plots to check the mode of inhibition and *Ki* value calculation.

***4.3.4 Antileishmanial effect of compounds*:** Antileishmanial effect of successful inhibitors were investigated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described earlier (*Mossman 1983; Saudagar et al. 2013*). Promastigote cells 1 x 10^6

cells were allowed to grow for 24 hours in presence of different concentrations of compounds. Cells were centrifuged and then after removing media, MTT (0.5 mg/ml) was added and incubated for 4 hours. Again centrifuge was done and DMSO added. Reading was taken at 570 nm. Amphotericin B (1 µM), a standard antileishmanial compound, was used as positive control. The reading of positive control was subtracted from experimental data values at different concentrations of test compounds.

***4.3.5 Estimation of Polyamine level by HPLC*:** Polyamines (putrescine, spermidine) levels of *L. donovani* promastigotes were measured using HPLC as described earlier (*Porta et. al., 1981; Enomoto et. al., 2006; Singh et. al., 2015*). *In vivo* polyamine concentrations were assessed after IC_{50} dose of M-2 and M-5 treatment. Briefly, 1 ml of *Leishmania* promastigotes (2.5 $x10^6$ cells ml^{-1}) was treated with M-2 and M-5 for 24 h. Equal numbers of untreated and treated promastigotes (1 x 10^6 cells) were used to compare the polyamine pools. Promastigotes were washed with PBS and centrifuged to collect cells. Subsequently, promastigotes were lysed in 40 µl phosphate buffer and polyamine levels were estimated. In brief, lysed promastigotes (40 µl) were used to derivatize polyamines by adding 120 µl of 75 mM dansyl chloride and 40 µl of saturated sodium carbonate, bringing the final volume of the derivatized lysates to 200 µl. Therefore, the polyamine levels represent the concentrations in cell lysates of 1 x 10^6 *Leishmania* promastigotes diluted to 200 µl in the process of derivatization. *In vivo* polyamine concentrations of untreated and treated promastigotes were compared.

***4.3.6 Expression level estimation of Spermidine synthase*:** *Leishmania* promastigote (1 ml) cells of density $1X10^7$ cells ml^{-1} were taken and treated with IC_{50} dose of compounds for 24 hours. Washed with PBS for two times and then mRNA was isolated and stored at -80°C till used, equal amount of mRNA was used and converted into cDNA using reverse transcriptase. Real time PCR was performed using Applied Biosystem 7500 software. α-tubulin was used for normalization of real-time PCR expression results. Comparative Ct ($\Delta\Delta$Ct) method was considered for analysis of relative gene expression level (*Livak et. al., 2001*).

***4.3.7 Estimation of decrease in the level of thiol by DTNB*:** Inhibition of *Ld*ODC may lead to decrease in reduced thiol level which is measured by microtiter plate assay using DTNB as explained earlier (*Shukla et al. 2011, Shukla et al. 2012*). In brief, promastigote culture (1ml) with cell density of 6 x 10^6 cells ml^{-1} was treated with *Ki* value of successful inhibitor of *Ld*ODC for different time intervals. Cells were centrifuged (1,000 x g for 5 min at 4°C) for harvesting. Cell pellet was dissolved in 20 mM Glycine-HCl buffer pH 2.5, sonicated for 10 min and centrifuged. Supernatant (100 µL) was taken in microtitre plate and 100 µL of 500 mM phosphate buffer pH 7.5 and 20 µL of DTNB (1mM) was added to each well. Absorbance at 412 nm was measured.

***4.3.8 Measurement of reactive oxygen species in Leishmania promastigotes*:** Intracellular ROS levels were measured in presence of successful inhibitors of *Ld*ODC using CM-H_2DCFDA (5-(and -6)-chloromethyl-2, 7-dichlorodihydro-fluorescein diacetate acetyl ester) dye as explained earlier (*Saudagar et al. 2013; Shukla et al. 2011; Saudagar et al. 2011, Shukla et al. 2012*). In brief, *Leishmania* cells were treated with IC_{50} value that is several folds higher than *Ki* value of *Ld*ODC inhibitors for different time intervals. Subsequently, *Leishmania* cells were centrifuged, washed and re-suspended in 10 mM Phosphate Buffer Saline pH 7.4. These cells were incubated with 10 µM CM-H_2DCFDA probe in dark for 45 mins. Reactive oxygen species levels were measured as an increase in fluorescence due to conversion of non-fluorescent dye to highly fluorescent 2', 7' dichlorofluorescein with an excitation at 488 nm and emission at 530 nm by Spectrophotometer and Fluorescence microscope both.

4.4 Results and discussion

***4.4.1 Inhibition study of Leishmania donovani ornithine decarboxylase* (*Ld*ODC):** Out of all compounds tested, three compounds were found to inhibit recombinant *Ld*ODC. The inhibition study data for each of the individual compound was fitted to Lineweaver- Burk plots for analysis. Compound M-2 is found to be uncompetitive inhibitor as V_{max} and K_m both were decreased after addition of 100 µM of inhibitor without changing the slope of

Lineweaver- Burk plots (Figure 4.1A). The calculated *Ki* value for compound M-2 is 79.73 µM whereas, compound M-5 is a non-competitive inhibitor which is depicted by decrease in V_{max} in presence of 100µM of inhibitor concentration. In presence of compound M-5, K_m of enzyme remains unchanged (Figure 4.1B). Calculated *Ki* value for the same is 370.63 µM. Effect of mangiferin on *Ld*ODC is shown in Figure 4.1C. The mode of inhibition is found to be non-competitive which is depicted by decrease in V_{max} in presence of 50 µM of inhibitor concentration (Figure 4.1C). The calculated value of *Ki* for mangiferin is 107.57 µM. The *Ki* values of the inhibitors are taken as the average of three independent inhibition experiments. Among all successful inhibitors, compound M-5 has highest *Ki* value. The compound is found to be the least effective inhibitor. Other two compounds, mangiferin and compound M-2 shows moderate inhibition. *In silico* identified inhibitor that have shown experimental inhibition with *Ld*ODC are listed in table 1 along with docking statistics.

***4.4.2 Interaction mode of successful inhibitor with LdODC*:** *In silico* identified inhibitor that have shown experimental inhibition with *Ld*ODC are listed in table 4.1 along with docking statistics. These inhibitors are analyzed for their interaction with *Ld*ODC (Figure 4.2). This study showed that all the three inhibitors bind around the active site of *Ld*ODC. In case of M-2 compound, Lys 33, Asp52, Arg120, Lys135 and Glu 243, are making hydrogen interaction with inhibitor whereas Ala 77, Phe 132 are showing hydrophobic interaction. In case of M-5 compound, Lys 33, Lys 135, and Thr 134 are making hydrogen interaction while His 163, Phe 306 are making hydrophobic interaction. Compound mangiferin is also interacting with *Ld*ODC by making hydrogen interaction with Glu 58, Ser 166, Arg 246, Asn 302 and Asp 307. It is worth mentioning that initial 255 residues of *Ld*ODC (N-terminal extension) were excluded in generation of the model in our previous report (*Chakraborty et al., 2013*) so residue 256 in sequence corresponds to residue 1 in the modelled structure.

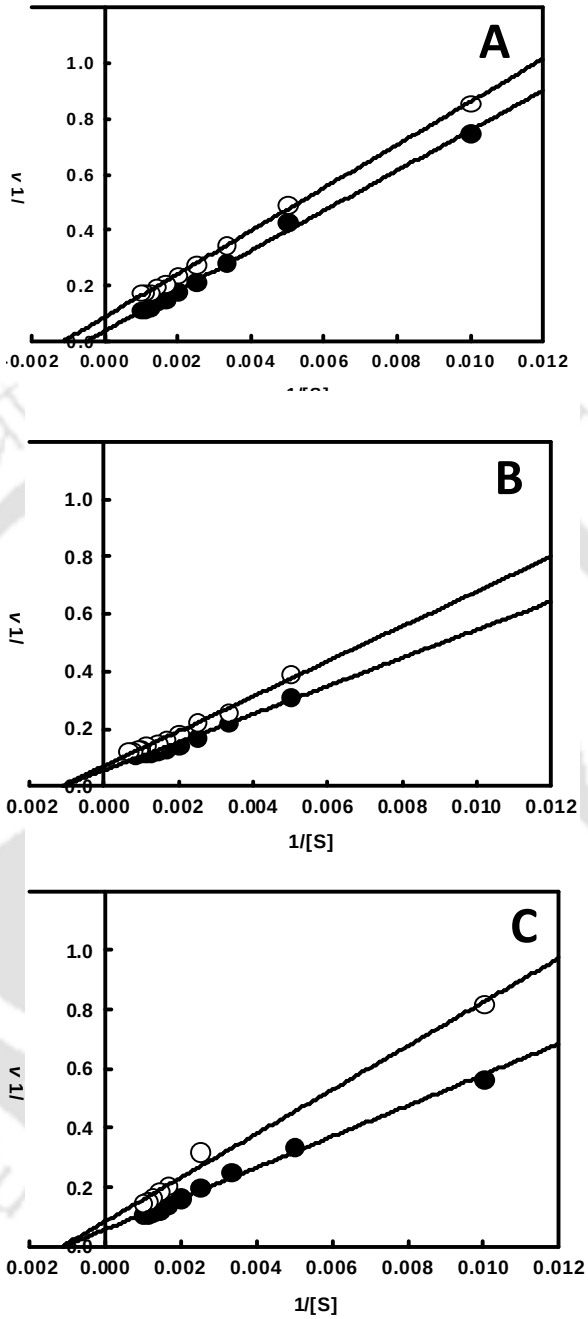
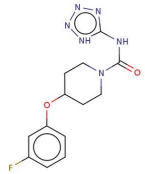
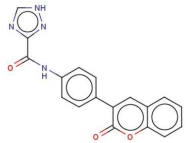
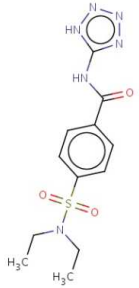
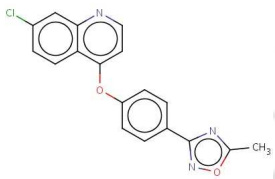
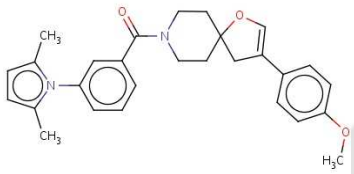
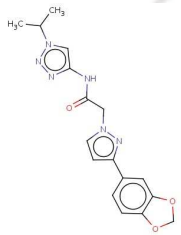
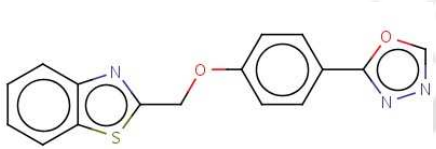
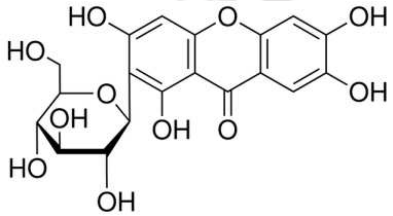


Figure 4.1: Enzyme inhibition studies: **(A)** Inhibition of ornithine decarboxylase by compound M-2. Close circle without inhibitor, open circle with inhibitor of 100 µM concentration. As V_{max} and K_m both changes and slope of Lineweaver-Burk plot remains same in presence of inhibitor, the mode of inhibition is uncompetitive **(B)** Inhibition of ornithine decarboxylase by compound M-5. Close circle without inhibitor, open circle with inhibitor of 100 µM concentration. As V_{max} is changing but K_m remains same in presence of inhibitor, the mode of inhibition is non-competitive. **(C)** Inhibition of ornithine decarboxylase by compound mangiferin. Close circle without inhibitor, open circle with inhibitor of 50 µM concentration. As V_{max} is changing but K_m remains same in presence of inhibitor, the mode of inhibition is non-competitive.

Table 4.1: Docking Parameter of successful screened compounds which were used in inhibition study.

Sl.No.	Compound ID/ Name/Structure	Free energy (Human ODC) Δ*G* Kcal/mol	Free energy of *Ld*ODC Δ*G* Kcal/mol	Difference in Δ*G* Kcal/mol
1	M1/ 4-(3-fluorophenoxy)-N-(1H-1,2,3,4-tetrazol-5-yl)piperidine-1-carboxamide/ 	-8.64	-10.16	1.52
2	M2 / N-[4-(2-oxo-2H-chromen-3-yl)phenyl]-1H-1,2,4-triazole-3-carboxamide 	-8.77	-9.87	1.10
3	M3/ 4-(diethylsulfamoyl)-N-(1H-1,2,3,4-tetrazol-5-yl)benzamide 	-8.07	-9.60	1.53

4	M4/ 7-chloro-4-[4-(5-methyl-1,2,4-oxadiazol-3-yl)phenoxy]quinoline 	-8.51	-9.46	0.95
5	M5/ 8-[3-(2,5-dimethylpyrrol-1-yl)benzoyl]-3-(4-methoxyphenyl)-1-oxa-8-azaspiro[4.5]dec-2-ene 	-8.38	-9.36	0.98
6	M6/ 2-[3-(2H-1,3-benzodioxol-5-yl)pyrazol-1-yl]-N-(1-isopropyl-1,2,3-triazol-4-yl)acetamide 	-7.79	-9.28	1.49

7	M7/ 2-[4-(1,3,4-oxadiazol-2-yl)phenoxymethyl]-1,3-benzothiazole 	-8.00	-9.24	1.24
8	Mangiferine/ 1,3,6,7-Tetrahydroxyxanthone C_2-β-D-glucoside 	7.23	9.32	2.03

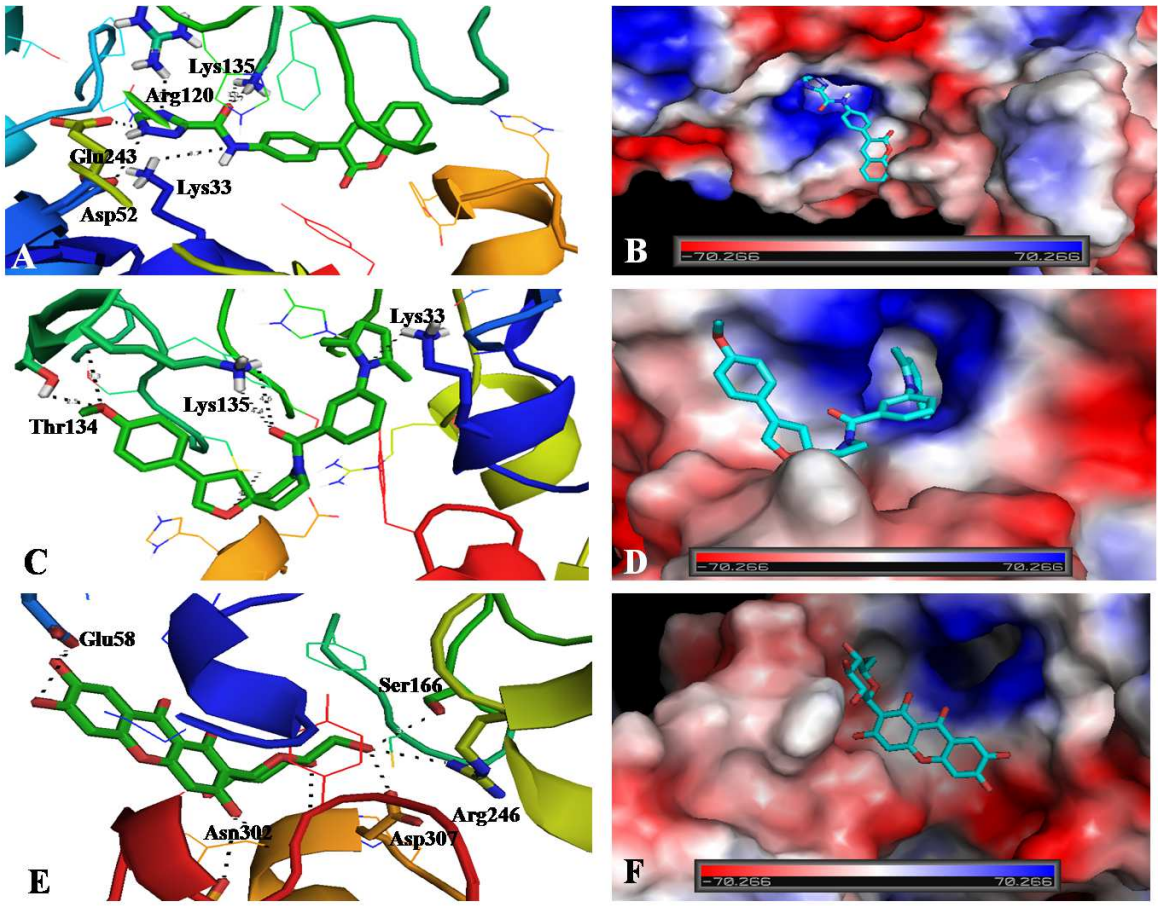


Figure 4.2: Diagrammatic representation showing interaction of **(A)** M-2 compound, **(C)** M-5 compound, and **(E)** mangiferin compound with *Ld*ODC. The compounds are interacting with some of the main amino acids which are involved in making active site of the enzyme. Vacuum electrostatics (qualitative) surface representation is also shown for *Ld*ODC in the region where inhibitors **(B)** M-2 compound, **(D)** M-5 compound, and **(F)** mangiferin are interacting. Positive and negative potentials are coloured in blue and red, respectively.

***4.4.3 Antileishmanial activity assay*:** All compounds that have shown inhibitory effect on recombinant *Ld*ODC were tested for antileishmanial effect on *L. donovani* promastigotes cells using MTT cell proliferation assay. The IC_{50} value for compound M-2 was found to be 350 µM (Figure 4.3A). Leishmanicidal effect on promastigotes stage by compound M-5 was also evaluated (Figure 4.3B). The IC_{50} value for compound M-5 was found to be 125 µM. The IC_{50} values for the mangiferin tested against *L. donovani* promastigotes was calculated by plotting percentage cell viability *vs.* concentration and were found to be 950 µM shown in

Figure 4.3C. In the case of all inhibitors of *Ld*ODC, except compound M-5, IC_{50} values were several folds higher than *Ki* values. In case of compound M-5, IC_{50} values was less then *Ki* value making it most effective anti-leishmanial compound.

***4.4.4 Intracellular Putrescine and Spermidine level detection by HPLC*:** As *Ld*ODC is involved in synthesis of putrescine, *in vivo* inhibition of the enzyme should decrease putrescine. *Leishmania* promastigotes were incubated with IC_{50} doses of *Ld*ODC inhibitors and putrescine level were measured as mentioned in method section. Further as putrescine is used for synthesis of spermidine, level of spermidine was also estimated in the same condition. As shown in Figure 4.4A, putrescine concentration was decreased by almost 40% by both compounds; M-5 has shown comparatively more effect. The decrease in putrescine concentration confirms target specificity of the inhibitors. In case of compounds M-2 and M-5, decrease in the spermidine level was not as such as it was expected from decrease in putrescine level. The decrease in spermidine level was only ~20% compared to decrease in putrescine level ~40% (Figure 4.4B). In case of DFMO, specific inhibitor of ODC, decease in putrescine and spermidine levels were almost equal to ~50%.

***4.4.5 Expression level estimation of Spermidine synthase*:** Treatment of *Ld*ODC novel inhibitors identified in the study do not decrease spermidine level as much as it was expected from decrease in putrescine level. Standard inhibitor of ODC, DFMO has same effect on *in vivo* spermidine and putrescine levels. To understand molecular basis of difference in the effects, spermidine synthase expression after treatment of these compounds were studied. As shown in figure 4.5, treatment of compound M-5 has up-regulated the gene by two fold. Compound M-2 has also shown up-regulation of gene. DFMO has no effect on the expression of spermidine synthase gene.

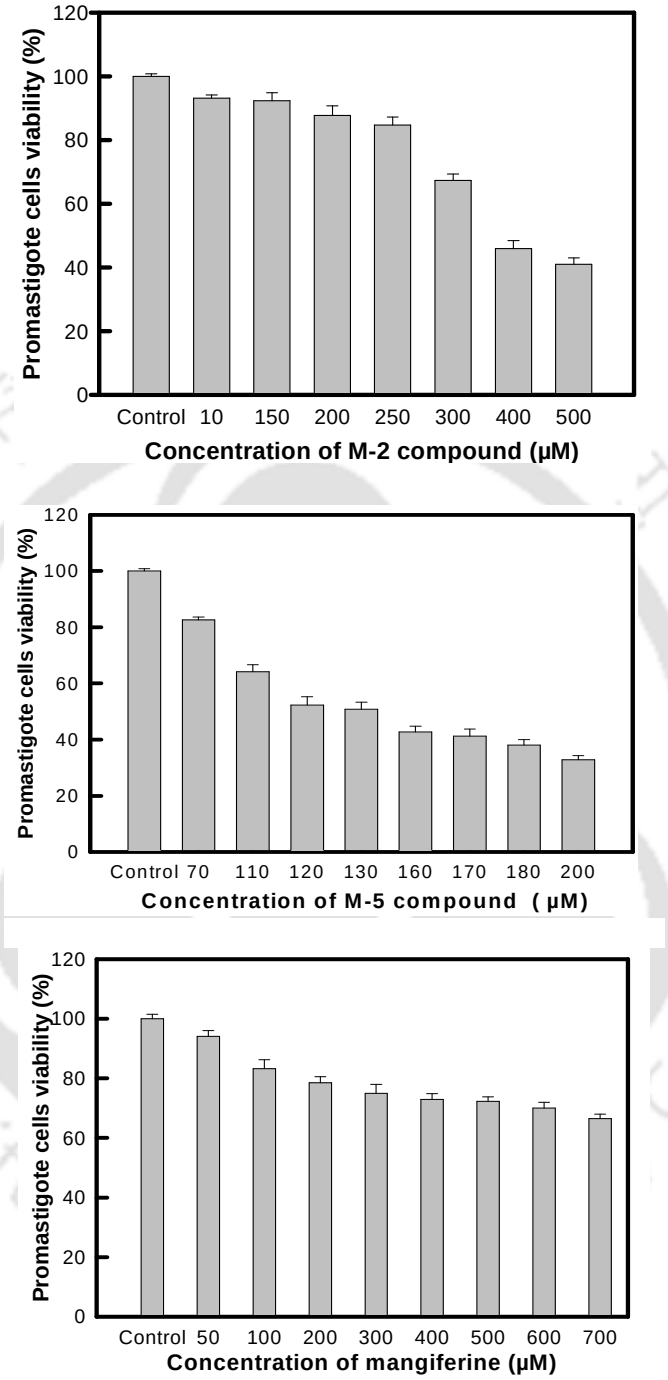


Figure 4.3: (A) Leishmanicidal effect on promastigotes stage by compound M-2 using MTT cell proliferation assay. The IC_{50} value for compound M-2 was found to be 350 µM. **(B)** Leishmanicidal effect on promastigotes stage by compound M-5, IC_{50} value for the same was found to be 125 µM. **(C)** The leishmanicidal efficiency was assayed using Mangiferin by MTT cell proliferation assay, the IC_{50} value found to be 950µM. Values are the average of three independent experiments.

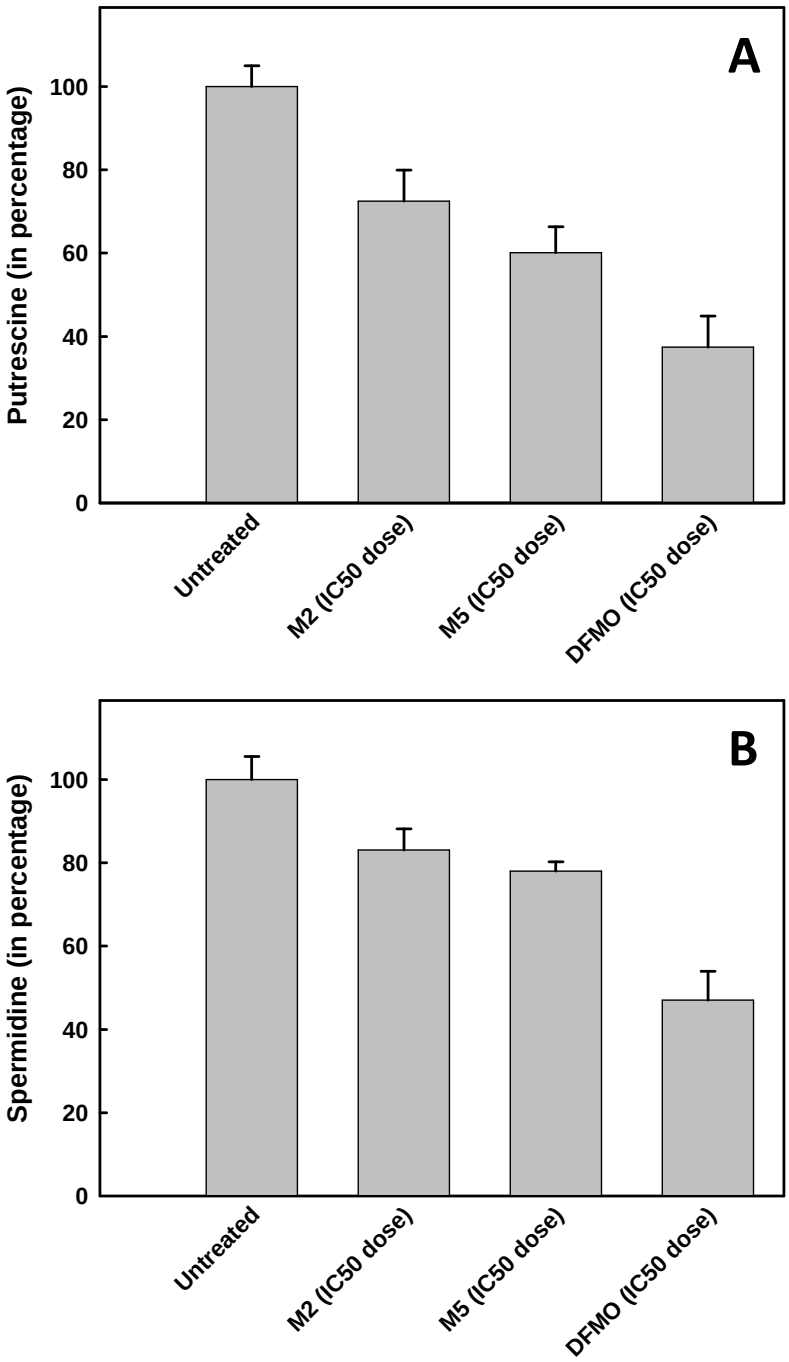


Figure 4.4: Putrescine and Spermidine level detection of promastigote by HPLC: (A) putrescine level in treated cells was decreased by 40% after treatment; (B) spermidine level decreased by 20% after IC_{50} treatment. DFMO was used as positive control; decrease in putrescine and spermidine level was more in case of DFMO than M-2 and M-5 compound.

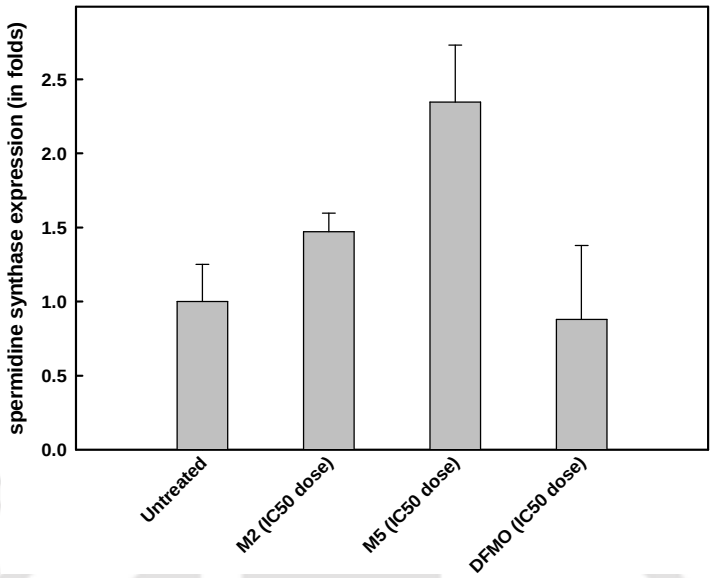


Figure 4.5: Expression level of spermidine synthase level increased by two fold after M-5 compound treatment and one and half fold by M-2 compound treatment. No change in expression level was seen after DFMO compound treatment.

***4.4.6 Estimation of decrease in the level of thiol by DTNB*:** Representative inhibitor, compound M-2 and mangiferin compounds have shown decrease in total free thiol as estimated by DTNB assay, which indicates decrease in intracellular thiol level of *Leishmania*. Compound M-2 treated *Leishmania* promastigotes for 24 hrs has shown decrease in thiol level by almost 27% (Figure 4.5A) and mangiferin has shown decrease in thiol level by 30% within 3 hrs. However, in case of mangiferin treated cells, regain of thiol level by 10 % was observed after next 3hrs (Figure 4.5B).

Ornithine decarboxylase (ODC) is involved in thiol (glutathione and trypanothione) biosynthesis (*Krauth-Siegel and Comini 2008; Rai et al. 2013*). So inhibition of ODC may lead to depletion of thiol level. The results further conforms target specificity of the compounds. However, interestingly, compound M-5 do not show any change in thiol concentration.

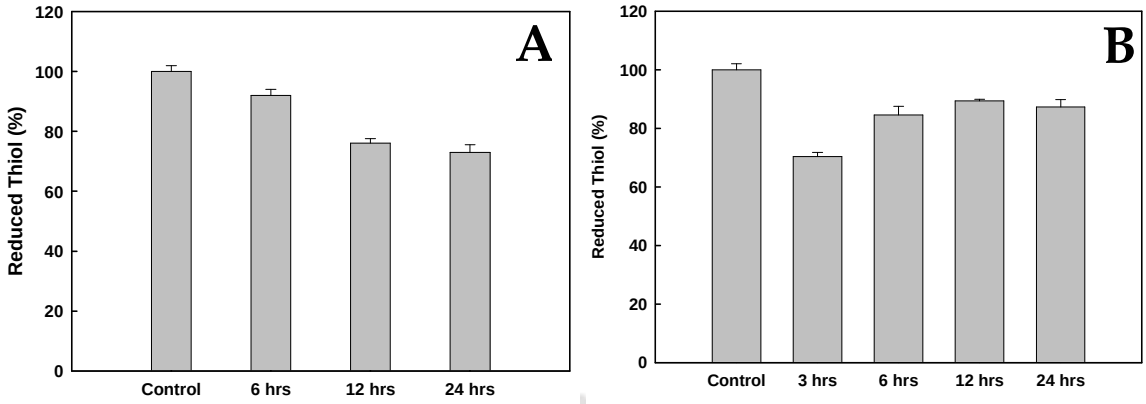


Figure 4.6: Estimation of free (reduced) thiol after IC_{50} value of **(A)** M-2 compound treatment for different time intervals, **(B)** Mangiferin. Free thiol was estimated by DTNB assay. Untreated promastigotes *Leishmania* cells was used for control.

***4.4.7 Measurement of reactive oxygen species in Leishmania promastigotes*:** As the inhibitors compounds have changed *in vivo* concentrations of putrescine and spermidine levels as well as total reduced thiol, they may disrupt redox homeostasis of the parasite. The reactive oxygen species (ROS) level is monitored with the help of a cell permeable dye called DCF. More the ROS generation more will be fluorescence. Increase in ROS level was recorded after treatment of *Leishmania* promastigotes for different time scale with M-2 (IC_{50} dose of inhibitor concentration) and mangiferin (of 100µM) compounds separately (Figure 4.6). Florescence microscopic images showing increase in ROS level after treatment of M-2 compound (Figure 4.6A). Florometric reading has shown M-2 compound treatment increased ROS level by around ~18 % after 24 hours, although half the level of it was attained after 12 hrs (Figure 4.6B). Mangiferin treatment has shown increased ROS level of upto ~11% in 3 hrs which did not increase after further treatment (Figure 4.6C). Surprisingly, compound M-5 fails to generate ROS.

***4.4.8 In vitro cytotoxicity on macrophage cell*:** The human macrophage cell line U937 was made adherent by using 100 ng/ml phorbol-12-myristate-13-acetate (PMA) treatment for overnight. Effect of compounds on the cell line is determined by MTT method. Data represent the mean ± SD of three independent experiments. There was no significant toxicity

shown by the compounds on the cells even at higher concentration up to 500 µM, indicating the compounds selectively inhibit *Leishmania* cells (Figure 4.8).

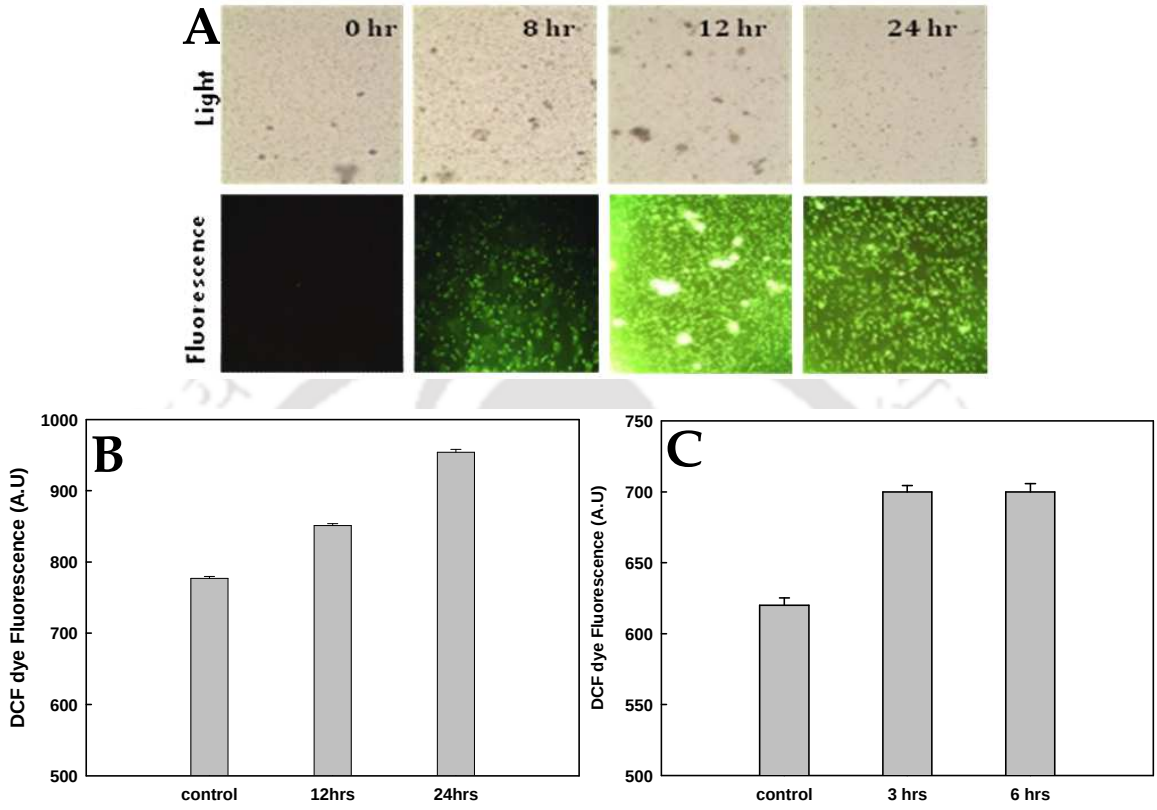


Figure 4.7: Reactive oxygen species (ROS) analysis by **(A)** Fluorescent microscopic images: M-2 compound treated *leishmania* with the help of H_2DCF dye (cell permeable), which becomes florescent after exposing with ROS; **(B)** Fluorometric readings after treating with M-2; **(C)** Fluorometric readings after mangiferin treatment.

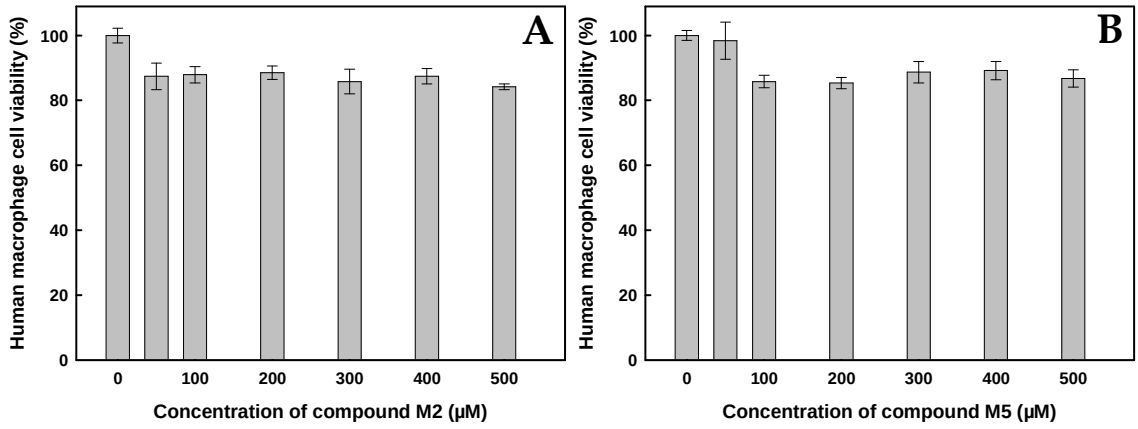


Figure 4.8: Cytotoxicity assay: The compounds have shown no cytotoxicity towards human macrophage cell line **(A)** Compound M2, **(B)** Compound M5.

4.5 Conclusion

In silico studies have shown good interaction of top hit compounds with active site of *Ld*ODC enzyme. Systemic screening of inhibitory effects of *in silico* identified compounds against functionally active recombinant ornithine decarboxylase from *L. donovani* confirms inhibitory action of three *in silico* predicted compounds. Compounds M-5 has shown better anti-leishmanial activity but it fails to generate ROS and there is no change in level of reduced thiol level. Compound M-2 and mangiferin has shown some effect on ROS and thiol level. Only minor effect on redox balance of pathogen, even at concentration of inhibitor that is several folds higher than *Ki* value, indicates existence of other mechanism to compensate inhibition of *Ld*ODC. The spermidine synthase expression level has up-regulated by these compounds, due to which decrease in spermidine is not as much as it is expected based on effect on putrescine concentration. The mechanism of over expression of spermidine synthase by these compounds remains elusive. It is worth mentioning that another inhibitor of *Ld*ODC, DFMO is not able to over-express spermidine synthase indicating that decrease in putrescine concentration is not solely responsible for the over expression. The most effective anti-leishmanial inhibitor identified in the study, compound M-5, decreases putrescine (~40 %) and spermidine (~20%) and shows over expression of spermidine synthase by two fold. However, it fails to show any effect on ROS, and reduced thiol. This shows alteration in some other gene expression to maintain reduced thiol concentration. Further, anti-leishmanial effect of *Ld*ODC inhibitor, compound M-5, is unrelated to ROS stress. Overall, the studies have identified differential effect of DFMO, well characterized inhibitor of ornithine decarboxylase, and three novel inhibitors indentified in the current studies. These detail of molecular mechanism of the differential effect yet to be deciphered.

CHAPTER V

Novel leads against miltefosine unresponsive *Leishmania donovani*.*

5.1 Abstract

Viceral leishmaniasis is an endemic deadly disease. There are few drugs against leishmaniasis but have numerous side effects. Unresponsiveness of only available oral drug miltefosine posses a big challenge for the therapeutics of the disease. Combination therapy is an important mode of treatment for various types of diseases including infectious diseases. Combinatorial therapy may be a better option which has lower side effects and less occurrence of drug resistance problem. We report two novel molecules, PS-203 (4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester) and mytomycin C, as very effective against miltefosine unresponsive strain of the parasite. Further, combinations of PS-203, mytomycin C with miltefosine were also evaluated that shows promising results against miltefosine unresponsive strain.

Keywords: Leishmaniasis; Drug resistance; Drug combinations

*Part of the review is communicated for publication.

5.2 Introduction

Available drugs of Leishmaniasis suffer from high toxicity and drug resistance. Failure of miltefosine, the only oral drug, especially in India is a big threat (*Croft, 2006*). Combination of available drug needs to be considered apart from discovery of novel drug candidates effective on available drug unresponsive strain (*Maltezou, 2009*). Combination of imiquimod and meglumine antimonite for cutaneous leishmaniasis treatment reduced the amount of meglumine antimonate needed, reduced both the time of treatment and the occurrence of drug resistance due to antimony based therapy (*Arevalo et al., 2001*). Further, combination of miltefosine and paromomycin has proved to be the most cost effective treatment strategy in India in recent times (*Meheus et al., 2010*). Combinatorial therapy is an important mode of treatment for various types of diseases including infectious diseases. To decrease the duration of therapy, a combination of two or more effective anti-leishmanial compounds would be a better idea. As shown in figure 5.1, combination therapy has several advantages.

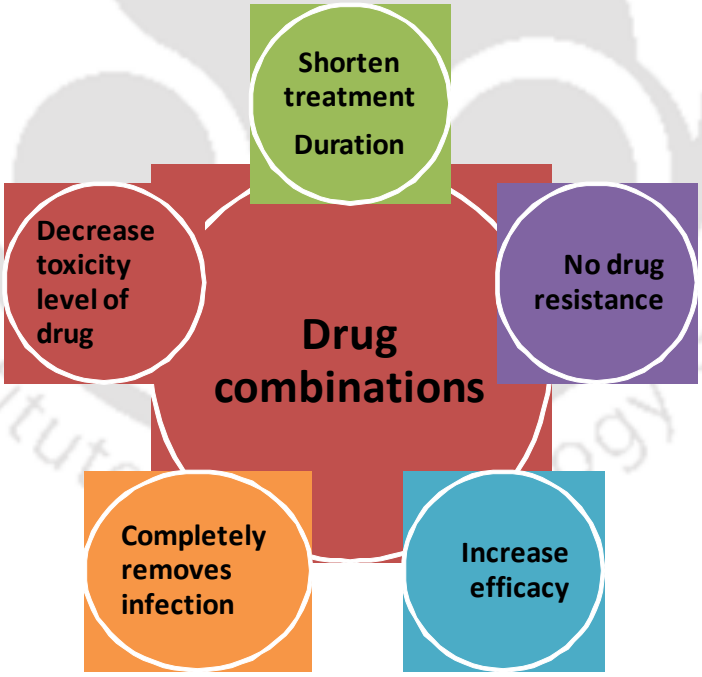


Figure 5.1: Schematic representation showing benifits of drug combinations used for treatment of leishmaniasis: increases the efficacy of drug, no report of occurance of drug resistance, decrease of duration of treatment, decrease toxic effect of drug etc.

Several other combinations of the drug against treatment of the parasite were reported to be more efficient (Table 5.1). However, not much attempts have been made against miltefosine unresponsive strain

Table 5.1: List of various drug combinations used against leishmaniasis:

	Drugs in combinations	**References**
1	Sodium stibogluconate + Paromomycin	*Jha, 2006*
2	Interferon gamma + SbV	*Jha, 2006*
3	Amphotericin B + Miltefosine	*Jha, 2006*
4	Miltefosine + Paromomycin	*Freitas-Junior et al., 2012*
5	Amphotericin B + Paromomycin	*Griensven et al., 2010*
6	Miltefosine + Sodium stibogluconate	*Seifert et al., 2007*
7	Miltefosine + Sitamaquine	*Seifert et al., 2007*
8	Rifampin + Quinolones	*Seifert et al., 2007*
9	Amphotericin B + BSO + DFMO	*Purkait et al., 2012*
10	Amphotericin B + Verapamil	*Purkait et al., 2012*
11	Azoles + allopurinol	*Bryceson et al., 2001*

Our group has earlier reported oxabicyclo derivative, PS-203 (4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester), as good anti-leishmanial agent with minimum toxicity, both *in vitro* and *in vivo* models (*Saudagar et al., 2013; Saudagar et al., 2014*). In this report, we have evaluated efficacy of PS-203 compound against miltefosine unresponsive strain. Further, combinations of PS-203 with other anti-leishmanial compounds were tested against miltefosine unresponsive strain.

5.3 Materials and Methods

***5.3.1 Parasites, cell lines and chemicals*:** The *Leishmania donovani* (MHOM/IN/2010/BHU1081) and miltefosine unresponsive *Leishmania donovani* (BHU-1155) was obtained from Prof. Shyam Sundar, Banaras Hindu University, India and cultivated in M199 liquid media supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. BHU-1155, a miltefosine unresponsive isolates, obtained after a month of miltefosine treatment from patient (18 years old male with no past history of the disease) spleenic material. The procedure is optimized and working in our laboratory (*Saudagar et al. 2013*; *Shukla et al. 2011*; *Saudagar et al. 2011*; *Shukla et al. 2012*).

All other chemicals used in the experiments were of the highest grade procured from Sigma-Aldrich or Merck.

***5.3.2 Anti-leishmanial effect of compounds*:** Anti-leishmanial effect of successful inhibitors were investigated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described earlier (*Mossman 1983; Saudagar et al. 2013*). Promastigote cells 1 x 10^6 cells were allowed to grow for 24 hours in presence of different concentrations of compounds. Cells were centrifuged and then after removing media, MTT (0.5 mg/ml) was added and incubated for 4 hours. Again centrifuge was done and DMSO added. Reading was taken at 570 nm. Amphotericin B (1 µM), a standard anti-leishmanial compound, was used as positive control. The reading of positive control was subtracted from experimental data values at different concentrations of test compounds.

5.4 Results and discussion

Miltefosine responsive and unresponsive strains were grown in our lab and sensitivity towards miltefosine was reconfirmed by MTT based method (Figure 5.2). The data re-confirms that the strain is miltefosine unresponsive in laboratory condition as well. We tested effects of various compounds on miltefosine unresponsive strain. Recently a report from our lab has proved that oxabicyclo derivative (for simplicity we named it as PS-203) has proved

to be an efficient anti-leishmanial agent as it causes apoptosis by generating ROS, changes mitochondrial membrane potential, and inhibits redox enzymes (TryS and TryR) in wild type strain of *Leishmania* (*Saudagar et al., 2013*). Another report proved that the compound has shown no *in vivo* toxicity in mice. *In vivo* studies on hamster model further confirmed anti-leishmanial effect of the compound (*Saudagar et al., 2014*). Effect of PS-203 was also evaluated on miltefosine unresponsive *Leishmania donovani* (BHU-1155) as well as miltefosine responsive *Leishmania donovani* (BHU-1081) in similar laboratory condition for a comparison (Figure 5.3). The data indicates that compound PS-203 is equally effective against miltefosine unresponsive strain with IC_{50} value of 4.0 ± 0.5 µM.

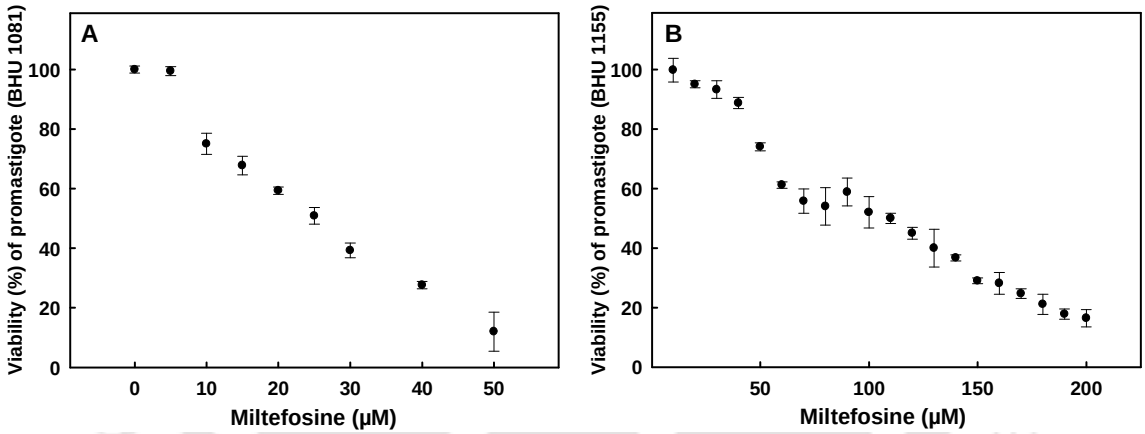


Figure 5.2: Miltefosine treatment to **(A)** wild type *L. donovani* promastigote *Ld*1081. The estimated IC_{50} value for *Ld*1081 strain was 25 µM **(B)** miltefosine unresponsive strain *L. donovani* promastigote *Ld*1155. The IC_{50} value for *Ld*1155 strain is 100 µM. The data confirms that the *Ld*1155 strain is miltefosine resistant strain.

We have also explored the combination of PS-203 with miltefosine in both miltefosine responsive and unresponsive strain of *Leishmania* (Figure 5.4). The data shows that PS-203 shows improved efficacy in combination with miltefosine. The IC_{50} value of PS-203 was >1 µM. Interestingly as shown in figure 5.4A, 25 µM miltefosine without PS-203 (0 µM on x-axis) can kill only 60% *Leishmania* promastigote but in the presence of 1 µM PS-203 efficacy is significantly higher. Similarly, combination of PS-203 with miltefosine has decreased the promastigote viability of unresponsive strain improving efficacy of PS-203 (Figure 5.4B). Combination of 25 µM miltefosine with 2.5 µM PS-203 has decreased the

promastigote viability. The viability further decreased with increase in miltefosine concentration.

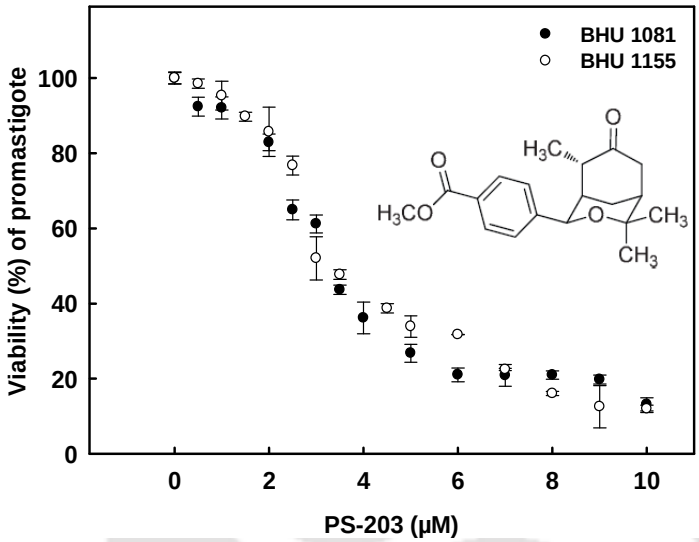


Figure 5.3: Effect of PS-203 compound on *Ld*1081 and *Ld*1155 strains of *L. donovani*. The data indicates that the compound is equally effective on both strains. An IC_{50} value of PS-203 against *Ld*1081 and *Ld*1155 strains is 4.0±0.5 μM which is marginally low compared to our earlier studies (*Saudagar et al., 2013*). Chemical structure of PS-203 compound (4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester) is shown in inset of Figure. Experiments are done in triplicate and data shown is average of three independent experiments.

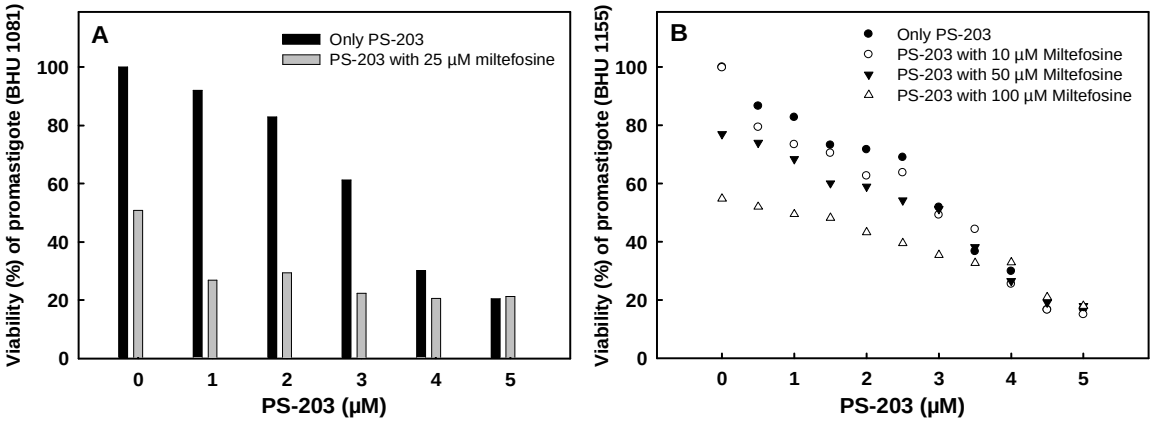


Figure 5.4: Miltefosine and PS-203 combination on miltefosine unresponsive strain of *L. donovani* promastigote (BHU 1155) as well as miltefosine responsive strain of *L. donovani* promastigote (BHU 1081) **(A)** PS-203 shows significantly improved efficacy in presence of miltefosine against Miltefosine responsive strain **(B)** miltefosine unresponsive strain treated in presence of PS-203 alone and in combination with PS-203 with miltefosine (10 μM, 50 μM, 100 μM). The combination of drug has shown improved efficacy. Experiments are done in triplicate and data shown is average of three independent experiments.

Another report also proved that mitomycin C is also a potential anti-leishmanial agent (*Shukla et al., 2011*). Therefore, we checked the effect of the compound on miltefosine unresponsive strain and our data proved that mitomycin C is also equally effective towards miltefosine unresponsive strain (Figure 5.5). Toxicity issue related to mitomycin C limits its applications hence if we employ this compound in combination with other effective compounds then it will reduce the toxic effect of mitomycin. A significant inhibition of miltefosine unresponsive strain was observed after using the cocktail of mitomycin C, miltefosine and PS-203 (Figure 5.6).

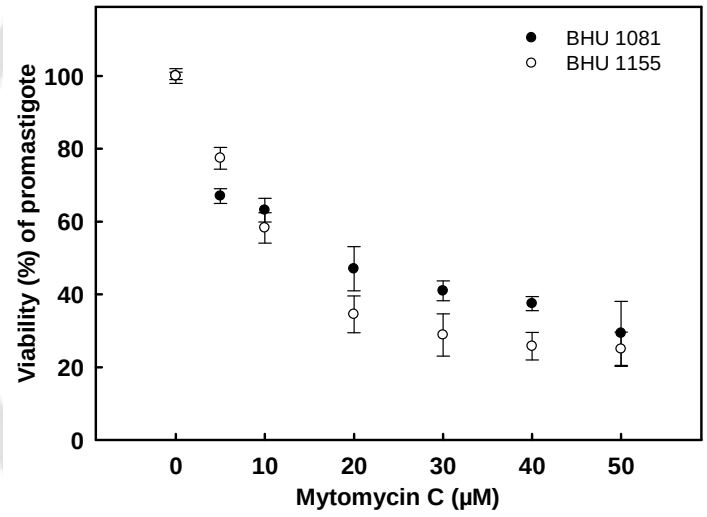


Figure 5.5: Effect of mitomycin C on *Ld*1081 and *Ld*1155 strains of *L. donovani*. The compound is marginally more effective on *Ld*1155. IC_{50} Values of mitomycin C against *Ld*1081 and *Ld*1155 strains are 15.03 μM and 12.8 μM, respectively.

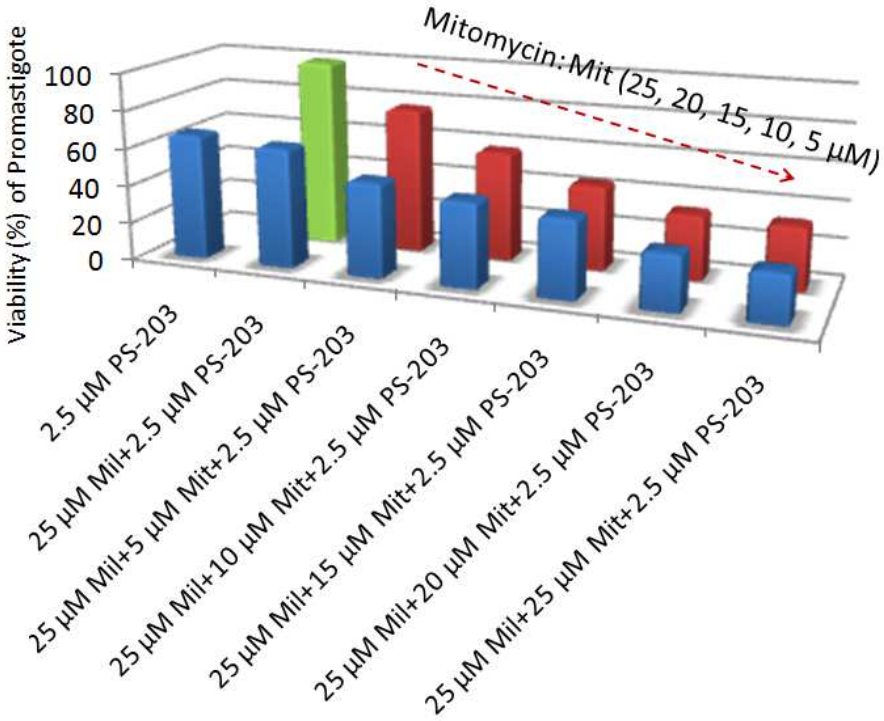


Figure 5.6: Combinations of Mitomycin C, Miltefosine and PS-203 were eveluated for effect on milefosine unresponsive strain of *Leishmania* promastigote. The bar with green color (■) indicates 25 µM miltefosine treatment.

5.5 Conclusion

Control of worldwide spread of leishmaniasis is challenged by emergence of drug resistance, which is a matter of concern. Changing response of *Leishmania* towards existing drugs necessitates continuous discovery of new drugs in future. Combinatorial regimen would be more effective in resistant strain because of its multi-target property. Similarly, novel drugs may also be useful. Miltefosine (hexadecylphosphocholine) was a major breakthrough for treatment of leishmaniasis. Miltefosine unresponsiveness is a greater challenge in the management of the disease. We have identified PS-203 compound as very effective against miltefosine unresponsive strain. Further, our data suggests that combination of PS-203 and miltefosine could be a great strategy against both Miltefosine unresponsive as well as Miltefosine responsive *Leishmania donovani*. Combination of miltefosine, PS-203 and

mytomycin C has shown more encouraging result suggesting a good combinatorial regimen to treat miltefosine resistant strain (Figure 5.7).

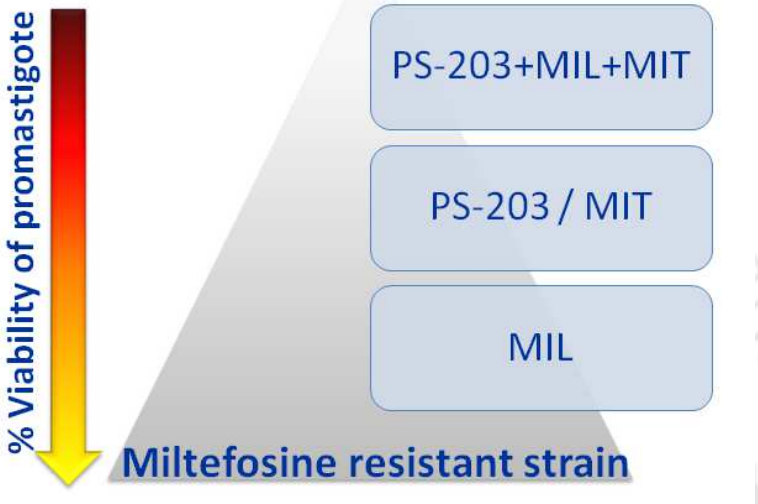


Figure 5.7: Schematic representation showing decrease in mitefosine unresponsive promastigote viability by treatment of PS-203 compound and mytomycin C separatly. Combination of miltefosine, PS-203 and mitomycin C has shown significant decrease in promastigote viability, suggesting a good combinatorial regimen to treat miltefosine resistant strain of *Leishmania*.

SUMMARY AND FUTURE SCOPE

Significant findings are listed below:

1. Microarray experiments has shown list of up-regulated and down-regulated genes in miltefosine unresponsive strain compared to miltefosine responsive strain. For my Ph.D. work, I selected the processes involved in oxidative metabolism of parasite. We have found that miltefosine unresponsive strain accumulates a ROS under conditions that are sufficient enough to cause ROS-induced mitochondrial membrane potential damage, apoptotic death and release of Ca^{2+} of miltefosine responsive *Leishmania*. Unresponsive strain has also shown up-regulated redox enzymes compared to responsive strain.

2. Cloned, expressed and purified full length and truncated (without N-terminal extension) form of ODC. I have reported that T-*Ld*ODC is more stable than *Ld*ODC. Detailed biochemical and biophysical characteristics of the enzyme (full length and truncated form) were analysed to understand role of N-terminal extension. The data suggest that the N-terminal extension helps in folding of the enzyme in active conformation at the cost of activity, and this is a good example of activity stability trade off.

3. Further, using *in silico* computational method we have identified three novel inhibitors of *Ld*ODC, which has shown good interaction with active site of the enzyme. The effect of compounds are tested on recombinant enzyme and then on the parasite. One compound has shown better anti-leishmanial activity, but surprisingly it fails to generate ROS and there is no change in level of reduced thiol level. Rest two compounds have shown some effect on ROS and thiol level. Only minor effect on redox balance of pathogen, even at concentration of inhibitor that is several folds higher than *Ki* value, indicates existence of other mechanism to compensate inhibition of *Ld*ODC. The spermidine synthase expression level has up-regulated by these compounds, due to which decrease in spermidine level is not as much as it is expected based on effect on putrescine concentration. The mechanism of over expression of spermidine synthase by these compounds remains elusive and these detail of molecular mechanism yet to be deciphered. This part of the work is submitted for publication.

4. We report a novel molecule PS-203 (4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester) and mitomycin C can be a good anti-leishmanial agent against miltefosine unresponsive strain of *Leishmania*. Combination of miltefosine, PS-203 and mitomycin C further decreased the promastigote viability to a significant level and this may be an effective strategy to tackle miltefosine resistant strain.

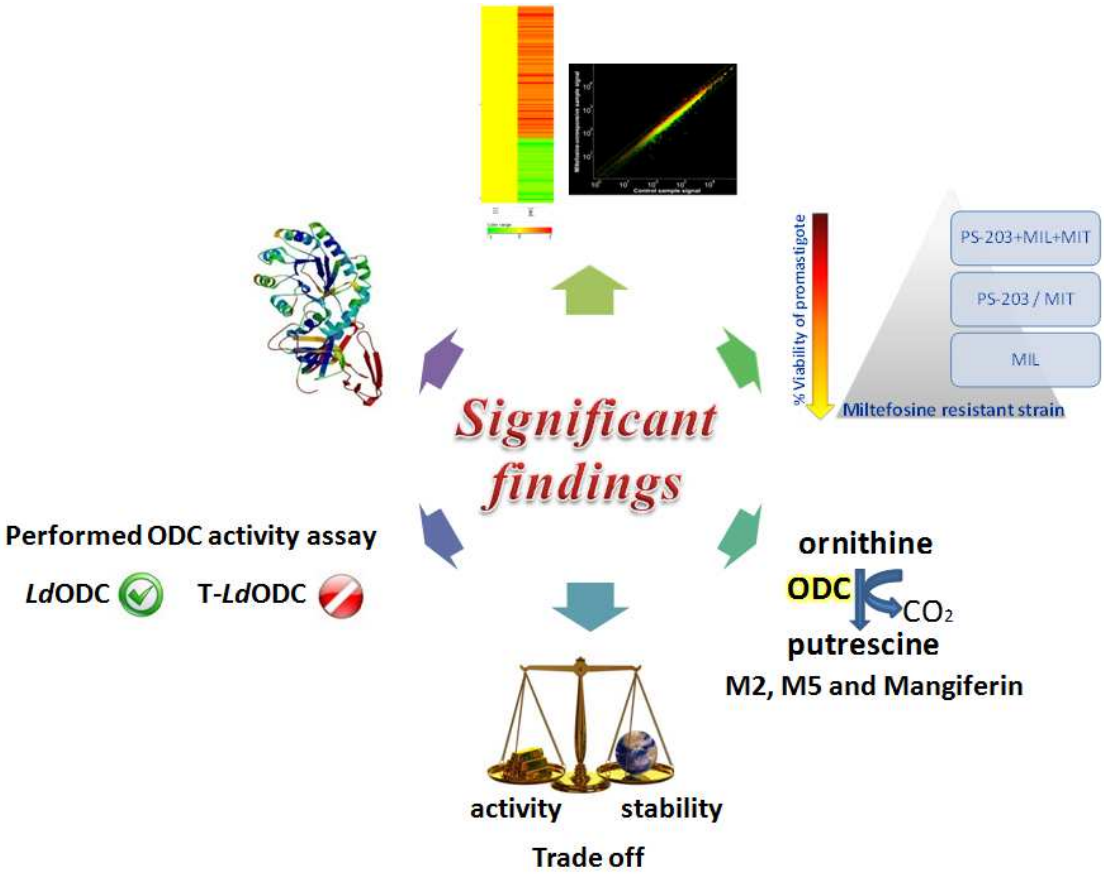


Figure: Schematic representation showing summary of Ph.D research work and prominent findings.

Forward Thinking

Based on the observation of our results we can plan some new experiments:

- Detail structural analysis of *Ld*ODC and T-*Ld*ODC can be done by using X-ray crystallography to get further insight into structural basis of activity-stability trade off property of the enzyme.

- Microarray data can be further compared with Real time PCR data, to identify drug potential targets for therapeutic intervention of Leishmaniasis caused by miltefosine resistant strain.

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A. Publications in Peer Reviewed Journals

1. **Mousumi Das**, Prakash Saudagar, Shyam Sundar and Vikash Kumar Dubey (2013) Miltefosine unresponsive *Leishmania donovani* has better ability of resist reactive oxygen species, *FEBS Journal*, 280:4807–4815.

2. **Mousumi Das**, Ritesh Kumar and Vikash Kumar Dubey (2015) Ornithine decarboxylase of *Leishmania donovani:* Biochemical properties and possible role of N-terminal extension, *Protein & Peptide Letters*, 22:130–136.

3. **Mousumi Das**, Shalini Singh and Vikash Kumar Dubey (2015) Novel inhibitors of ornithine decarboxylase of *Leishmania* parasite (*Ld*ODC): The parasite resists *Ld*ODC inhibition by over-expression of spermidine synthase (*Submitted on 14 April 2015, Molecular and Biochemical Parasitology*).

4. **Mousumi Das**, Gundappa Saha, Anil Saikia and Vikash Dubey (2015) Novel leads against miltefosine unresponsive *Leishmania donovani*. (*Submitted on 21 April, Antimicrobial Agents and Chemotherapy short communications*).

5. Sudipta Hazra, Subhalakshmi Ghosh, Madhushree Das Sarma, Smriti Sharma, **Mousumi Das**, Prakash Saudagar, Vijay Kumar Prajapati, Vikash Kumar Dubey, Shyam Sundar, Banasri Hazra (2013) Evaluation of diospyrin and its derivatives as antileishmanial agents and potential modulators of ornithine decarboxylase of *Leishmania donovani*, *Experimental Parasitology*, 135:407–413.

6. Neha Sharma, Anil Kumar Shukla, **Mousumi Das** and Vikash Kumar Dubey (2012) Evaluation of plumbagin and its derivative as potential modulator of redox thiol metabolism of *Leishmania* parasite, *Parasitology Research*, 110:341–348.

7. Shalini Singh, Shyamali Sarma, Shashank Katiyar, **Mousumi Das**, Ruchika Bhardwaj, Durai Sundar, and Vikash Kumar Dubey (2015) Probing molecular mechanism of hypericin induced parasitic death: An insight into role of spermidine beyond redox metabolism of *Leishmania*, *Antimicrobial Agents and Chemotherapy*, 59:15–24.

8. **Mousumi Das**, Shalini Singh, Ruchika Bhardwaj and Vikash Kumar Dubey (2015) Neglected tropical disease Leishmaniasis: a brief over view (*Bulletin*).

B. Publications in conference Proceedings

1. **Mousumi Das** and Vikash Kumar Dubey. Exploring ornithine decarboxylase for novel drugs against *Leishmania donovani*. 80th Annual Meeting of Society of

Biological Chemists, held at Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India, November 12-15, 2011.

2. **Mousumi Das** and Vikash Kumar Dubey. Discovery of novel drug candidate by targeting ornithine decarboxylase enzyme: Biochemical analysis of cellular effects of identified drug candidates. Participated in "BioSangam-2013, International conference on Health, Environment & Environmental Biotechnology" organized by Motilal Nehru National Institute of technology. [**Best Oral Presentation Award**].

3. **Mousumi Das** et al., 2013. Activities of *Leishmania* research group at IIT Guwahati. Participated in "TECHEVINCE 1.0", conducted by IITG Technical Board, 14th November, 2013.

4. **Mousumi Das** and Vikash Kumar Dubey. Actively participated in Research Conclave from 23rd – 26th March, 2015 organized by the PhD Council of the Students' Academic Board (SAB), Indian Institute of Technology Guwahati.

C. Workshop / training attended

1. Participated in 13th INDO-US Cytometry Workshop on 'Application of Flow Cytometry in Nanotechnology and Plant Genomic', jointly organized by Department of Biotechnology, IIT Guwahati, India & Miller School of Medicine, University of Miami, USA during 8th-10th October, 2012 held at IIT Guwahati.

2. Participated in "Bioinformatics and its implications", short term training held at Bioinformatics Infrastructure Facility (BIF), Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028 from 15th to 21st December 2008. Funded by Department of Biotechnology, Govt. of India, New Delhi.

3. Participated in The National Conference on "Recent Advances in Cancer Biology and Therapeutics-2014" organised by Department of Biotechnology, IIT Guwahati held on December 5th, 2014.

4. Participated in the Symposium cum Workshop on "Advances in Computational Biology and Computer Aided Drug Design" held at IIT Guwahati, organized by the Bioinformatics Infrastructure Facility (BIF), Department of Biosciences and Bioengineering during 24th-26th June, 2015.