Understanding the molecular mechanisms implicated in resistance of *Leishmania donovani* to miltefosine: A proteomics approach

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

By

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University of Hyderabad

(A Central University established in 1974 by act of parliament)

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"DECLARATION"

I, **T.S. Jalajaveronica Rani**, hereby declare that this thesis entitled **"Understanding the molecular mechanisms implicated in resistance of *Leishmania donovani* to miltefosine: A proteomics approach"** submitted by me is based on the results of the work done under the guidance and supervision of **Dr. Radheshyam Maurya** at Department of Animal Biology, School of Life Sciences, University of Hyderabad. The work presented in this thesis is original and plagiarism free. I also declare that no part or in full of this thesis has been submitted previously to this University or any other University or Institution for the award of any degree or diploma.

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"CERTIFICATE"

This is to certify that this thesis entitled **"Understanding the molecular mechanisms implicated in resistance of *Leishmania donovani* to miltefosine: A proteomics approach"** is a record of bonafide work done by **Ms. T.S. Jalajaveronica Rani**, a research scholar for the Ph.D. programme in the Department of Animal Biology, School of Life Sciences, University of Hyderabad, under my guidance and supervision. The work presented in this thesis is original and plagiarism free. The thesis has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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T. S. Jalajaveronica Rani

Dedicated to Almighty and my family

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ABBREVIATIONS	
βME	β-Mercaptoethanol
ABC transporter	ATP binding cassette transporter
AcCN	Acetonitrile
ACR2	Arsenate reductase 2
ALAS1	Aminolevulinic acid synthase 1
ALM	Autoclaved *L. major*
ALP	Alkaline phosphatase
AmB	Amphotericin B
ATCC	American Type Culture Collection
Anti-His	Anti-Histidine
BCDO2	Beta carotenoid dioxygenase
BCG	Bacillus Calmette-Guerin
Bcl2	Beclin 2
BOD	Biochemical Oxygen Demand
BSA	Bovine Serum Albumin
BZRAP1	Benzodiazepine receptor associated protein 1
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CIAPIN1	Cytokines induced apoptosis inhibitor 1
CL	Cutaneous leishmaniasis
CpG ODN	CpG oligodeoxynucleotide(s)
CS	Citrate synthase
Ct	Threshold cycle
DALYs	Disability adjusted life years
DAPI	4',6-diamidino-2-phenylindole
DCF	2',7'-dichlorofluorescein
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eIF2	Elongation initiation factor 2
EPB	Electroporation buffer
EtBr	Ethidium bromide
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FeSODA	Iron superoxide dismutase A
FPP	Farnesyl pyrophosphate
FPPS	Farnesyl diphosphate synthase
G6P	Glucose 6-phosphate

GAA	Guanidinoacetic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATM	Glycine amidinotransferase
GFP	Green fluorescent protein
GO	Gene ontology
GP63	Glycoprotein 63
GTP	Guanosine triphosphate
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_3PO_4	Orthophosphoric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hh pathway	Hedgehog pathway
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRE	Heme responsive element
HSP70	Heat shock protein 70
i.d	Intradermally
IC_{50}	Half maximal inhibitory concentration
IEF	Isoelectric focusing
IFA	Immunofluorescence assay
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
iNOS	Inducible nitric oxide synthase
INPP4A	Inositol polyphosphate-4-phosphatase type I
IPG	Immobilized pH gradient
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCl	Potassium chloride
KD	Knockdown
kDa	Kilo Dalton(s)
KEGG	Kyoto Encyclopedia of Genes and Genomes
KIF1BP	KIF1 binding protein (or) Kinesin binding protein
kV	Kilovolt(s)
LAmB	Liposomal amphotericin B
LB broth	Luria-Bertani broth
lbs	Pounds per square inch
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDB	Leishman Donovan bodies
LdMT	*L. donovani* miltefosine transporter
LPG	Lipophosphoglycan
LZ	Leishmanization
MALDI TOF-TOF	MALDI- time of flight tandem mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
MCL	Mucocutaneous leishmaniasis
MDH2	Malate dehydrogenase 2
MDP	Muramyl dipeptide
MFI	Mean fluorescence intensity

$MgCl_2$	Magnesium chloride
MLX	Max-like protein X
MLXIP	MLX interacting protein
MPLA	Monophosphoryl lipid A
mRNA	Messenger ribonucleic acid
MRPA	Multi drug resistant protein A
ms	Milli second(s)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-dipheynyltetrazolium bromide
MWCO	Molecular weight cut off
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NC membrane	Nitrocellulose membrane
NF-κB	Nuclear factor kappa-ligght-chain-enhancer of activated B cells
NLRP3	NACHT, LRR, and PYD-domains containing protein 3
NNN medium	Novy-MacNeal-Nicolle medium
NO	Nitric oxide
NRD1	Nardilysin (N-arginine dibasic convertase)
NTD	Neglected Tropical Diseases
^oC	Degree Celsius
OD_{600}	Optical density at 600 nm
p.i.	Post infection
PARP1	Poly ADP-Ribose Polymerase 1
PBR	Peripheral type benzodiazepine receptor
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDI	Protein disulfide isomerase
pH	Potential of Hydrogen
PI	Propidium iodide
PI5P	Phosphatidylinositol 5-phosphate
PIK3C2A	Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 alpha
PIK3C2G	Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 gamma
PKDL	Post-kala azar dermal leishmaniasis
PM	Peritrophic matrix
PMSF	Phenylmethylsulphonylfluoride
PPG	Proteophosphoglycan
PpGalec	*Phlebotomus papatasi* galectin
PRP1	Pentamidine resistant protein 1
Rac1	Ras-related C3 botulinum toxin substrate 1
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Room temperature

Sb^{III}	Trivalent antimoniate
Sb^{V}	Pentavalent antimoniate
SC	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SLA	Soluble leishmanial antigen(s)
SNP	Single nucleotide polymorphism
Stat3	Signal transducer and activator of transcription 3
SYNJ2	Synaptojanin 2
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TCA cycle	Tricarboxylic acid cycle
TDR	Thiol dependent reductase
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoro acetic acid
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TPI	Triose phosphate isomerase
TPR	Trypanothione reductase
TXNIP	Thioredoxin interacting protein
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
VL	Visceral leishmaniasis
WHO	World Health Organization
WT	Wild type
XIAP	X-linked inhibitor of apoptosis
µF	Microfarad(s)
NP-40	Nonidet P-40
IL-12	Interleukin 12
KMP-11	Kinetoplastid membrane protein 11
IL-10	Interleukin 10
IL-4	Interleukin-4
PI(3)P	Phosphatidylinositol 3-phosphate
Egr-1	Early growth response 1
IL-1β	Interleukin 1 beta
PI(3,4)P2	Phosphatidylinositol 3,4-bisphosphate
PI(3,4,5)P3	Phosphatidylinositol3,4,5-triphosphate

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Chapter 1:

Introduction

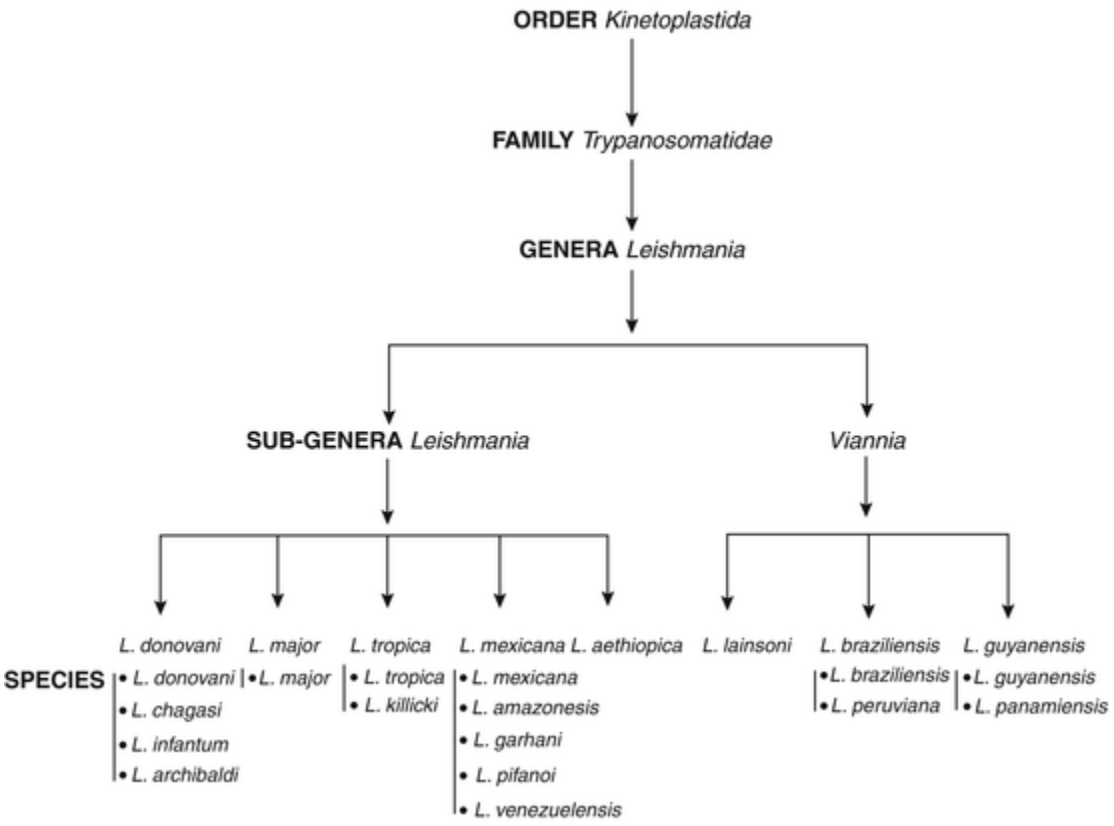
Parasites are the non-symbiotic organisms that live at the expense of their hosts. There are endoparasites and ectoparasites. Endoparasites exploit the host's resources necessary for their survival and gradually cushion themselves against the host's attacking immune system. Among the endoparasites, protozoan parasites are unicellular and can invade any cell in our body. The parasites of the genus *Leishmania* belong to such class. *Leishmania* parasite causes a spectrum of disease from self-healing to fatal Leishmaniasis. This life-threatening disease results in around 20,000-40,000 deaths per annum. Although thought to be the ninth major deadly disease; it has been classified as one of the neglected tropical diseases (NTDs).

1.1 History

Leishmaniasis has a long history dating back to the first century AD. The symptoms, typical of cutaneous and mucocutaneous leishmaniases, such as skin lesions and facial deformities were observed in the agricultural workers during 15^{th} and 16^{th} century. These ulcers were named, "white leprosy," "Andean sickness," or "valley sickness." In Africa and India, in the mid 18^{th} century, it was described as "kala-azar" or "black fever." In 1756, Alexander Russell discovered leishmaniasis in a Turkish patient and called it "Aleppo boil."

The disease "Leishmaniasis" was named after William Leishman, a Glaswegian doctor serving with the British Army in India, in 1901. He discovered ovoid bodies in the spleen of a British soldier, in Dum Dum town near Calcutta, who was experiencing bouts of fever, anaemia, muscular atrophy and swelling of the spleen. He described it as "Dum Dum fever" in 1903. A few weeks after Leishman's discovery, Charles Donovan also observed the similar symptoms in other kala-azar patients. These bodies in the patient's spleen were found using Leishman's stain and hence called Leishman-Donovan bodies after William Leishman and Charles Donovan. Sir Ronald Ross coined the term *Leishmania donovani* to this parasite after the names of both the doctors recognizing their contribution in its discovery (*Ross 1903*).

1.2 Classification



(*Mishra, Kishore et al. 2013*)

Fig 1.1 Classification of *Leishmania*

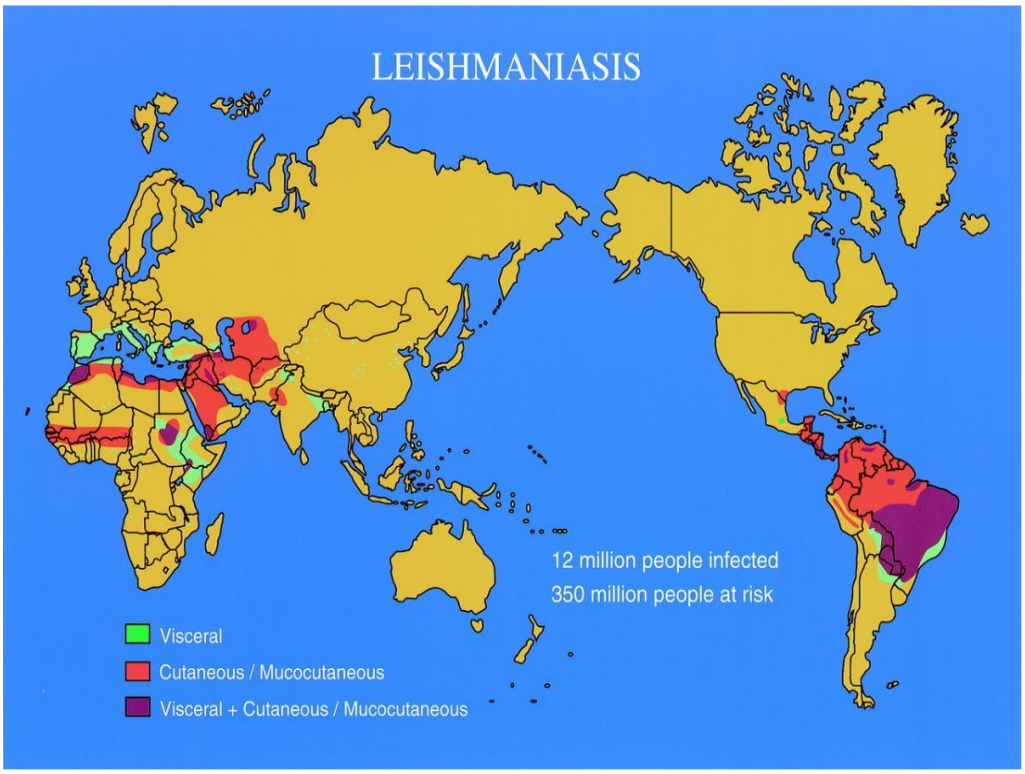
The parasitic species of the genus *Leishmania* are the unicellular protozoans. They are transmitted between the vertebrates by the female Phlebotomine sandflies (*Ready 2013*). They have been classified in the Kinetoplastida order, Trypanosomatidae family and the genera *Leishmania*. About 20 *Leishmania* species are known to infect mammals most of which are humans. The *Leishmania* species is again classified into two subgenera namely, *Leishmania* and *Viannia*. Most of the *Leishmania* species from the subgenus *Leishmania* cause human cutaneous leishmaniasis (CL). Human CL is predominantly caused by *L. major*, *L. mexicana*, and *L. braziliensis*. Few species namely *L. donovani*, *L. chagasi*, and *L. infantum* cause human visceral

leishmaniasis (VL). *L. tropica*, usually causing CL, is also known to visceralize to form Oriental Sores.

1.3 Global distribution

The leishmaniasis, one of the NTDs, is transmitted to humans and other mammals by the sandfly vectors (*Murray, Berman et al. 2005*). It can be caused by around 20 *Leishmania* species implicating a complex life cycle involving multiple arthropod vectors and mammalian reservoir species (*Ashford 1996; Ready 2013*). The transmission can be either vector-borne or non-vector borne. Vectors include sandflies belonging to *Phlebotomus* spp. (Old World) or *Lutzomyia* spp. (New World). Non-vector transmission, though rare, can occur through accidental laboratory infection, blood transfusion, or organ transplantation (*Cardo 2006*). Mammalian reservoirs encompass domestic dogs, rodents, sloths, and opossums. Transmission of the leishmaniases can be either anthroponotic or zoonotic.

The leishmaniases rank as the leading NTDs in terms of mortality and morbidity with an estimated 50,000 deaths in 2010 (*Lozano, Naghavi et al. 2012*) and 3.3 million disability adjusted life years (DALYs) (*Murray, Vos et al. 2012*). Leishmaniases are widely disseminated around the world ranging from intertropical zones of America, Africa, and extend in to temperate regions of South America, Southern Europe, and Asia. The geographical distribution depends on sandfly species, their ecology, and the internal developmental conditions of the parasite (*Desjeux 1996*). In case of VL, half a million new cases are estimated to arise annually, with more than 50,000 deaths circa. More than 90% of VL cases emerge in India, Nepal, Bangladesh, Sudan, Ethiopia, and Brazil (*Chappuis, Sundar et al. 2007*). While *L. donovani* infects children and adults, *L. infantum* and *L. chagasi* were found to affect infants, young children, and immunosuppressed individuals (*Maltezou 2008*). New World CL occurs in Mexico, Central America, and South America from Northern Argentina to Southern Texas and Southern Europe (*ul Bari 2006*). Old World CL is prevalent in Asia, Middle East, and Africa with the estimated annual incidence of 1-1.5 million cases. Over 90% of annual cases occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, and Syria *(Desjeux 2004; Assimina, Charilaos et al. 2008*) by *L. major* and *L. aethiopica*.

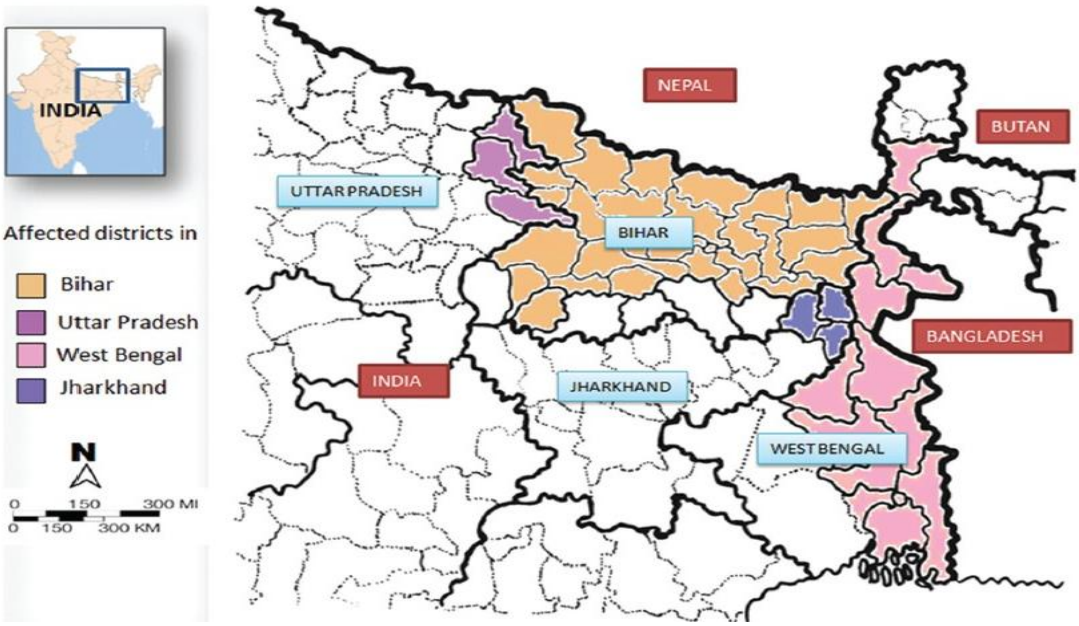


(*Handman 2001*)

Fig 1.2 Global distribution of Leishmaniasis worldwide

1.4 Visceral leishmaniasis in India

VL had re-emerged near eradication in the Indian subcontinent (*Bora 1999*). In India, the endemicity of seriously problematic life-threatening VL is confined to 52 districts of Bihar, Uttar Pradesh, Jharkhand, and West Bengal. The neighboring countries viz. Nepal (in 12 districts) and Bangladesh (in 45 districts) are also endemic to VL *(Sundar and Rai 2002; Alvar, Yactayo et al. 2006*). Bihar state in India records about 50% of the total burden in Indian subcontinent and is considered to be the "hotspot" of VL (*Singh, Reddy et al. 2006*). Recently, the neighboring country Bhutan has also joined the club of VL-affected areas (*Bhattacharya, Rinzin et al. 2010*). VL results in the loss of about 400,000 DALYs every year which approximately equals to the loss of US $ 140 million annually (*Joshi, Narain et al. 2008*).

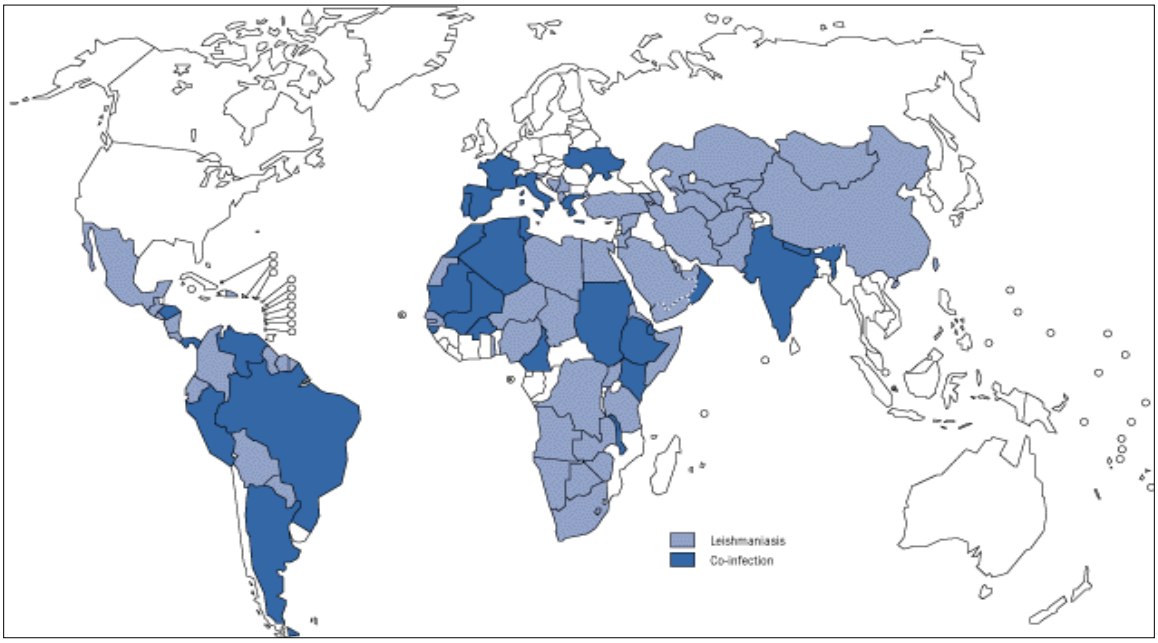


(*Muniaraj 2014*)

Fig 1.3 Epidemiology of VL in Indian sub-continent.

1.5 Co-infection with HIV

Besides the enormous VL cases, India ranks second largest HIV infected populous country in the world. The co-infection is a serious public health problem wherein HIV infection increases the risk of progression from asymptomatic infection to fatal VL and in turn VL accelerates HIV disease progression (*Sinha, Jha et al. 2010*). Even then, the number of new HIV infections per million in India had declined from 27 in 2000 to 12 in 2009 (*Muniaraj 2014*). The percentage of HIV infection was reported to be much less in VL patients with 1.5% compared to age and sex matched controls which showed 4.1% (*Thakur, Narayan et al. 2003*). Although there is a high burden of VL as well as HIV in the Indian subcontinent, the incidence of VL and HIV co-infection is still very limited (*Alvar, Yactayo et al. 2006; Mathur, Samantaray et al. 2006*). Till now, the HIV co-infection cases did not correlate with the number of VL cases.



(*Organization 2000*)

Fig 1.4 Global distribution of leishmaniasis and *Leishmania*-HIV co-infection, 1990-1998.

1.6 Clinical manifestations

Based on the clinical manifestations, the human leishmaniases are categorized into three forms namely; cutaneous, mucocutaneous and visceral leishmaniasis.

1.6.1 Cutaneous leishmaniasis

CL is associated with several species in the Old World and New World, viz. *L. major*, *L. tropica*, *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. peruviana*, and *L. guyanensis* (*Dedet, Pratlong et al. 1999; Banuls, Hide et al. 2007; Ameen 2010; den Boer, Argaw et al. 2011*). Though classically known to be visceralizing species, *L. infantum* also reported to cause benign cutaneous lesions in France (*Ponce, Ponce et al. 1991; Dedet, Pratlong et al. 1999*), *L. chagasi* in Central America (*Ponce, Ponce et al. 1991*), and *L. donovani* in Sri Lanka (*Karunaweera 2009*) and Yemen (*Pratlong, Bastien et al. 1995*). *L. donovani* produces a particular form of the cutaneous disease called post-kala azar dermal leishmaniasis (PKDL). Depending on the infecting species of *Leishmania (Hepburn 2003; Akilov, Khachemoune et al. 2007; Ameen 2010*)

and the human host factors (*Hide, Bucherton et al. 2007*), the CL can be either in localized or diffuse form. Usually, CL forms a dormant self-healing ulcer which leaves a permanent scar. Besides such ulcers, single crusted papules, verrucous or papulonodular ulcers are also formed. *L. mexicana* is known to cause necrosis of the external ear whereas *L. guyanensis* causes sporotrichoid lesions (*Ameen 2010; Goto and Lindoso 2010*). Other variants of CL symptoms are eczematoid, infiltrative plaques, paronychial, chancriform, erysipeloid, zosteriform, lupoid, whitlow, sprototrichoid, etc. (*Kubba, el-Hassan et al. 1987; Raja, Khan et al. 1998; Iftikhar, Bari et al. 2003; Karincaoglu, Esrefoglu et al. 2004; Bari and Rahman 2008; Bari and Ejaz 2009; Guimaraes, Machado et al. 2009; Padovese, Terranova et al. 2009*). In case of CL, all the exposed body parts are vulnerable to these lesions as they appear at the site of the sandfly bite. However, some reports state that the unusual locations like genital organs and palms have also been affected *(White and Hendricks 1982; Colebunders, Depraetere et al. 1999; Cabello, Caraballo et al. 2002; Alvar, Aparicio et al. 2008; Lindoso, Barbosa et al. 2009*). In some cases, multiple lesions are also observed which might be due to the multiple inoculations (*Shani-Adir, Kamil et al. 2005; Samaranayake, Dissanayake et al. 2008; Faye, Banuls et al. 2010*). Some rare cases exhibit CL as satellite lesions or it may progress around the site of the original lesion *(Kubba, el-Hassan et al. 1987; Kubba, al-Gindan et al. 1988; Calvopina, Uezato et al. 2006*) or may advance towards disseminated CL *(Kubba, al-Gindan et al. 1988; Grevelink and Lerner 1996; Murray, Berman et al. 2005; Goto and Lindoso 2010*). Disseminated CL is characterized by diffuse, non-ulcerated, non-healing nodular lesions which relapse after treatment and spreads to the whole body with remnant unattractive scars leading to death in some cases. This form of CL is caused by *L. aethiopica* in East Africa and *L. amazonensis* and *L. mexicana* in South America and has been associated with HIV co-infection (*Couppie, Clyti et al. 2004; Alvar, Aparicio et al. 2008*). CL often mimics other infectious diseases and cancers (*Zeegelaar and Faber 2008; Veraldi, Bottini et al. 2010*) leading to misdiagnosis and delay in starting treatment.

1.6.2 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (MCL) is one of the other clinical manifestations in the New World Leishmaniasis. It is also called as 'espundia.' It is usually caused by *L. braziliensis* and rarely by *L. panamensis*, *L. guyanensis*, and *L. amazonensis (Barral, Pedral-Sampaio et al.*

1991; Tojal da Silva, Cupolillo et al. 2006; Banuls, Hide et al. 2007; Ameen 2010; Guerra, Prestes et al. 2011). MCL is characterized by the extensive destruction of oronasal and pharyngeal cavities, occurrence of unsightly lesions and disfiguration of face. Besides nose; lips, mouth, pharynx, larynx, and middle ear are also affected (*Motta, Arruda et al. 2003; Lessa, Lessa et al. 2007; Motta, Lopes et al. 2007; Casero, Laconte et al. 2010*). Usually, the mucosal lesions are metastatic i.e. the cutaneous lesions gradually disseminate to form mucosal lesions *(Murray, Berman et al. 2005*). This dissemination may occur through the lymphatic system or by the direct contact between mucosa and cutaneous lesions (*Lessa, Lessa et al. 2007*). MCL is also observed in Sudan and North Africa of Old World. Some reports state the mucosal involvement of *L. infantum* (*Faucher, Pomares et al. 2011*).

1.6.3 Visceral leishmaniasis

VL is the most severe form of all types of leishmaniases. It is also called as Kala-azar and is predominant in the Indian sub-continent. It becomes fatal if left untreated. It is the second major disease after malaria and causes around 50,000 deaths per annum according to WHO (*Organization 2010*). It is usually caused by *L. donovani*, *L. infantum*, and *L. chagasi*. *L. donovani* is human specific and is responsible for high mortality in humans whereas the other two species *L. infantum* and *L. chagasi* are highly specific to dogs and rarely affect humans (*Seaman, Mercer et al. 1996; Murray, Berman et al. 2005; Nasereddin, Baneth et al. 2005; John, Dandona et al. 2011*). VL patients display the symptoms such as undulating fever associated with intermittent rigor and chills, fatigue, weight loss, hepatosplenomegaly, pancytopenia, and anaemia. Anaemia is caused by the persistent inflammatory state, hypersplenism, and bleeding. Some differences are observed in the symptoms of VL patients in various endemic areas. For example, the enlarged lymph nodes can be frequently observed in Sudanese VL patients whereas rarely noticed in the patients of Indian sub-continent (*Siddig, Ghalib et al. 1990; Zijlstra, el-Hassan et al. 1994*). The other symptom like hyper-pigmentation has been observed only in the patients of Indian sub-continent but not in the other areas. The derivation of name Kala-azar has been ascribed to this reason but the symptom of hyper-pigmentation has now become so rare or uncommon even in the Indian sub-continent which might be due to the prolonged illness as a consequence of unavailable treatment. The

splenomegaly increases as the disease progresses resulting in abdominal distension and pain (*Chappuis, Sundar et al. 2007*).

1.6.4 Post-kala azar dermal leishmaniasis

PKDL is a dermal complication, caused as a sequel to VL. It develops after a certain remission (ranging from weeks to several years) from infection in cured VL cases. In India, PKDL cases account for about 5-15% while in Sudan, it occurs in 50-60% (*Ramesh and Mukherjee 1995; Zijlstra and el-Hassan 2001*). The disease unfolds in various clinical forms from hypopigmented macules to infiltrated papules or nodules. Subsequent development of PKDL can be predicted by high interleukin 10 (IL-10) in the skin and peripheral blood and also elevated levels of C-reactive protein in the plasma of patients with VL (*Gasim, Elhassan et al. 1998; Gasim, Theander et al. 2000*).

PKDL became known in 1992 when Brahmachari confirmed eruption and plaque in the skin of cured VL patients as Leishman-Donovan bodies (LDB) in slit skin smear and termed it as dermal leishmanoid (*Brahmachari 1922*). Since the skin eruptions follow the kala-azar, it was renamed as post kala-azar dermal leishmaniasis. The features of PKDL vary in Sudan and in the Indian subcontinent. In India, PKDL emerges as a dermatosis with a remission of 2-3 years but it may occur much earlier of about after 6 months or much later of about 32 years. In 15-20% cases, subclinical infection is observed with no preceding history of VL *(Ramesh and Mukherjee 1995*). But, in Sudan, it occurs within first 6 months after the treatment in 50% of VL patients and in some cases during the treatment. About 8% of PKDL cases show no VL history whereas parallel VL and PKDL are reported in 18% cases *(Zijlstra, Musa et al. 2003*).

In the Indian subcontinent, the parasite is thought to be housed and disseminated by the untreated cases of VL and PKDL (*Thakur and Kumar 1992; Desjeux 2001*). Though the presence of the parasite in the skin of VL patients has been reported (*Napier 1946*), the parasites are predominantly found in the inaccessible organs. Whereas, PKDL features nodular skin lesions harboring LD bodies and are easily accessible to the vector, *P. argentipes*. Thus, the PKDL patients are thought to play a role in bridging the disease transmission in the absence of animal reservoir.

The clinical manifestations of PKDL vary from hypopigmented macules to infiltrated plaques and nodules. The three major representations of skin lesions include (i) monomorphic (macular and nodular), (ii) polymorphic or mixed (both macules and indurated lesions such as papules are present), (iii) rare presentations (for example, erythrodermic). In India, the nodules enlarge and form big plaques whereas in Africa, ulceration is common. Some unusual variations in the symptoms of PKDL also occur like annular, warty, papillomatous growth, fibroid with erythematous plaques and xanthomantous growth and presence of lesions in uncommon site such as eyelids, palms, and the perionychium. In most of the cases, PKDL persists as a chronic dermatosis. But complications arise when mucous membranes get affected and the corneal involvement leads to blindness (*Ramesh and Mukherjee 1995*). A report of nerve involvement in Indian PKDL suggests that it mimics leprosy clinically and pathologically *(el Hassan, Ghalib et al. 1992; Khandpur, Ramam et al. 2004*). Some reports state the emergence of PKDL in HIV patients from South America, Europe *(Rios-Buceta, Buezo et al. 1996; Ridolfo, Gervasoni et al. 2000; Bittencourt, Silva et al. 2003*), and India (*Nandy, Addy et al. 2004*). There is a possibility of VL recurrence after PKDL due to the immunosuppression caused by measles or malaria and tuberculosis (*Nandy, Addy et al. 1998*). Till date, the parasite or host factors are unknown that cause the emergence of PKDL after VL which ultimately causes a shift in the infection site from visceral organs to the dermis and also changes in the clinical manifestations.

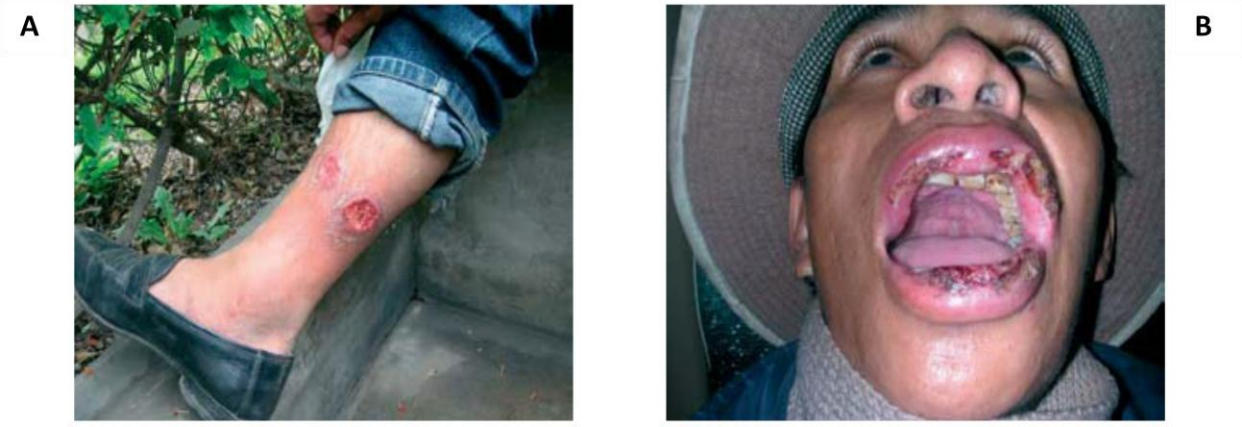


Fig 1.5 Clinical signs of Leishmaniasis. A) A patient from Peru with cutaneous leishmaniasis. B) A patient from Bolivia with mucosal leishmaniasis. C) A patient from Uganda with visceral leishmaniasis. D) A patient from India with nodular post-kala-azar dermal leishmaniasis (PKDL).

(*Chappuis, Sundar et al. 2007*)

1.7 Sandfly Vector

Order : **Diptera**

Class : **Insecta**

Family : **Psychodidae**

Phylum : **Arthropoda**

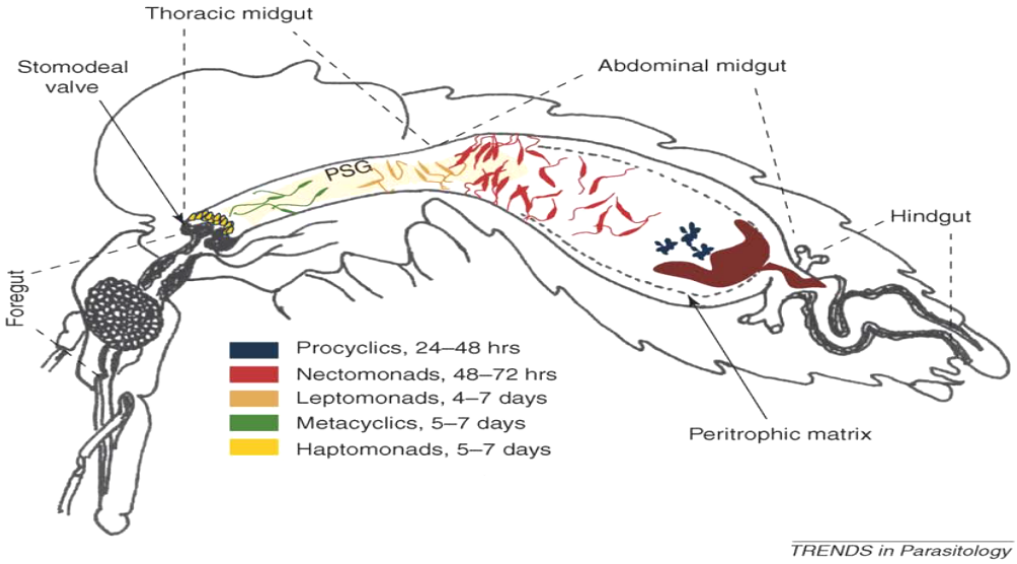
The leishmaniasis is transmitted by the dipteran fly called sandfly belonging to the phlebotominae subfamily which comprises of bloodsucking vectors of various diseases like leishmaniasis, bartonellosis, Phlebotomus fever, and vesicular stomatitis *(Tesh and Chaniotis 1975*). During the life cycle of sandflies, they metamorphose through the life stages larva, pupa and the adult. The immature stages complete their development in warm and moist environments like animal burrows. Hence, the sandflies are predominantly found at rodent habitations.

The sandflies obtain their carbohydrate nutrition from plant juices. In addition to this, females require at least one blood meal to complete their development. Most of the sandflies have been categorized in three genera namely, *Phlebotomus* and *Sergentomyia* of the Old World, and *Lutzomyia* of the New World. To date, over 800 species have been identified and categorized in five genera - *Phlebotomus*, *Sergentomyia* in the Old World and *Lutzomyia*, *Brumptomyia*, and *Warileya* in the New World. The proven vector species of *Leishmania* are classified into the genus *Phlebotomus* and *Lutzomyia* (*Munstermann 2004*). Majority of the species do not involve in the transmission of leishmaniasis. This might be because they do not feed on the blood of the reservoir animals or they may be incapable of supporting the development of *Leishmania* species *(Killick-Kendrick 1999; Munstermann 2004*). Less than 10% of sandfly species are thought to act as vectors of leishmaniasis among which only 30 species have been known to have the vectorial capacity. Each vector species can support the development and transmission of certain species of *Leishmania* (*Bates 2007*). In the suitable vector species, the *Leishmania* promastigotes attach to the gut epithelium, multiply and differentiate into the metacyclic forms infective to the mammalian host (*Killick-Kendrick 1999; Sacks 2001*). The attachment of promastigotes to the insect midgut is crucial (*Killick-Kendrick 1999; Sacks 2001*) which is mediated by lipophosphoglycan (LPG), the major surface glycoconjugate. The structures of LPG are polymorphic among species suggesting that LPG is the major determinant specifying a vector species (*Sacks, Modi et al. 2000; Sacks 2001*). The only midgut protein of sandflies shown to interact with *Leishmania* is PpGalec, a β-galactoside-binding lectin, found in *P. papatasi*, a principal vector of *L. major* in the Old World (*Kamhawi, Ramalho-Ortigao et al. 2004*). Genomic DNA hybridized with *PpGalec* was present in *P. papatasi* and *P. duboscqui*, both of which transmit *L. major* in nature, but absent in *P. sergenti* and *P. argentipes*, vector species of *L. tropica* and *L. donovani*, respectively, in the Old World, and *Lu. longipalpis* and *Lu. verrucarum* which transmit *L. infantum* and *L. peruviana*, respectively, in the New World (*Kamhawi, Ramalho-Ortigao et al. 2004*). The result strongly suggests that midgut molecules of sandflies are the key determinants of vectorial competency.

1.8 Life cycle in the vector

When a sandfly preys on the blood of mammals, the macrophages infected with amastigotes are also ingested along with the blood. The infected blood meal passes to the posterior abdominal midgut. The parasites migrate from posterior midgut to stomodeal valve during their life cycle and undergo different developmental stages. Each developmental stage undergoes morphological and functional changes helping in the survival of parasite inside the sandfly. Usually, the time taken for parasite development in the sandfly is about 6-9 days depending on the species. At first, the infected blood meal is contained by a peritrophic matrix (PM) within 4 h after reaching the posterior midgut. Here, the amastigotes are released into blood bolus and differentiate into small, sluggish procyclic promastigotes characterized by a short flagella. These are the forms that start their multiplication cycle in the sandfly and are separated from the midgut by a type I PM. These forms of parasites are resistant to the digestive enzymes of sandfly. These procyclic forms undergo rapid replication for the next 24-48 h. By day 2-3, these procyclics develop into large, motile, and slender nectomonads. At this point, the PM gets degenerated and the nectomonads escape into the gut lumen. There they anchor themselves to epithelial cells lining the migut and migrate forward to colonize the anterior thoracic midgut. From these forms, arise leptomonads by day 4. These are shorter and undergo the second multiplication cycle (*Rogers, Chance et al. 2002; Gossage, Rogers et al. 2003; Bates and Rogers 2004*). By day 5-7, the division of leptomonads results in a massive infection at the anterior midgut. At the final stage of parasite development in the stomodeal valve, two stages namely haptomonads and metacyclics are observed. It is still clearly not known whether the haptomonads arise from nectomonads or leptomonads. Haptomonads are highly specialized non-motile leaf-like forms with short flagella. They are attached to the cuticular lining of stomodeal valve and to each other, resulting in the concentric rings of parasites and form a parasite plug at the valve. The other forms called metacyclics are the infective stages for mammals. These non-dividing forms are found behind the stomodeal valve. These infective metacyclic forms are characterized by a small cell body with an elongated flagellum and are rapid, free-swimming forms resistant to complement-mediated lysis. Leptomonads produce a promastigote secretory gel, which fills the thoracic midgut, enveloping the leptomonads and metacyclics (*Rogers, Chance et al. 2002*). In heavily

infected sandflies, the stomodeal valve degenerates and a few metacyclics can pass through it into the foregut.



(*Kamhawi 2006*)

Fig 1.6 Life cycle of *Leishmania* in the sandfly vector

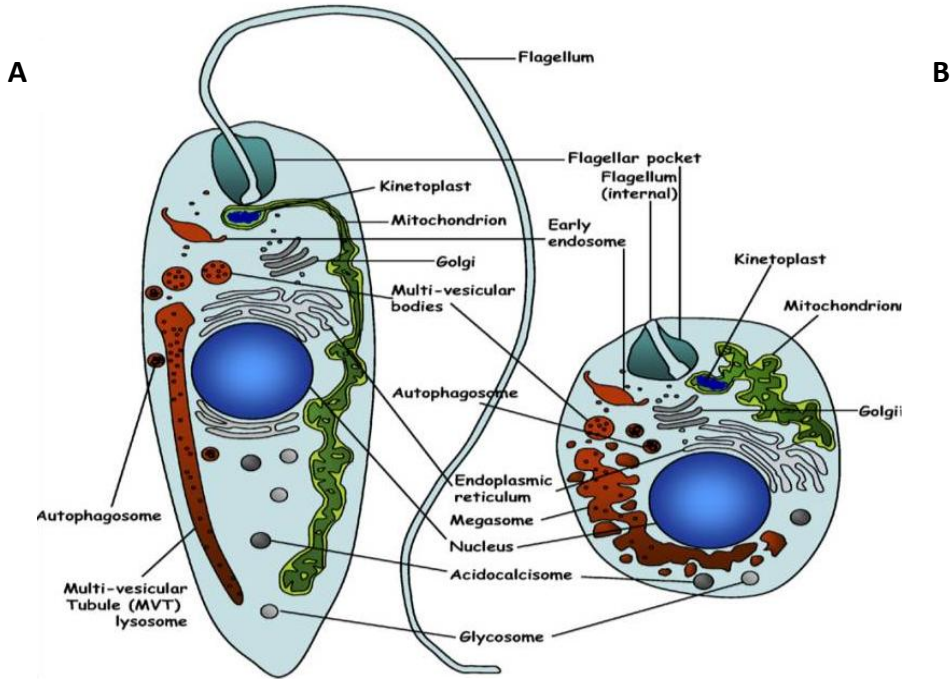
1.9 Morphology of *Leishmania*

1.9.1 Morphology of Leishmania promastigotes

Promastigotes are 15-30 µm in body length and 5 µm in width. It is extracellular, motile, and divides by longitudinal binary fission at 27^{o}C in the sandfly. They can be grown *in vitro* at 25^{o}C temperature on NNN medium, which has a solid phase of blood agar and a liquid phase containing a physiological salt solution.

1.9.2 Morphology of Leishmania amastigotes

Amastigotes are intracellular, non-motile forms in the vertebrate host. It divides by longitudinal binary fission at 37^{o}C. Intracellular amastigotes are 3-6 µm in length and 1.5-3.0 µm in width. The amastigotes are also called as LDBs. The flagellum is short and does not protrude beyond the body surface.



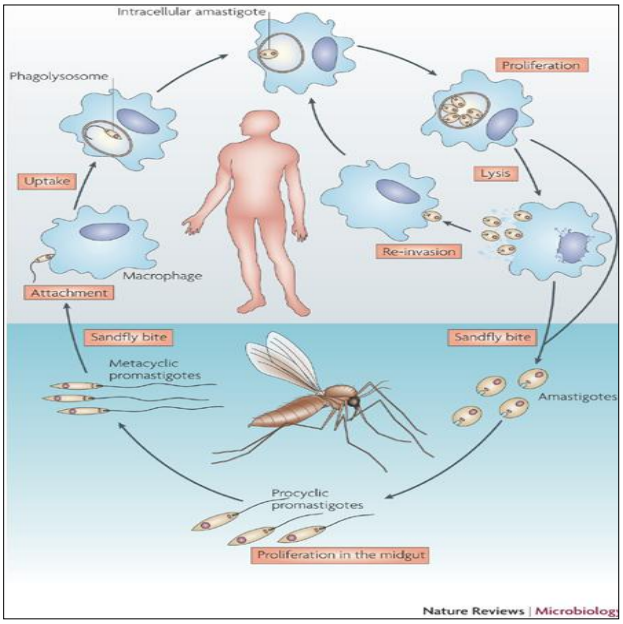
(*Besteiro, Williams et al. 2007*)

Fig 1.7 Ultrastructure of (A) promastigote form and (B) amastigote form

1.10 Life cycle in the human host

The life cycle of *Leishmania* begins with the female sandfly insect which is infected with *Leishmania* biting a human host. Following the ingestion of blood, the metacyclic promastigotes are released into the bite site by regurgitation. Thus released parasite reaches the mammalian skin and subsequently invades different cell types that it encounters such as dendritic cells, fibroblasts, neutrophils that are rapidly recruited to the bite site and mainly the macrophages. The promastigote form of the parasite is attached to the surface of macrophage. The process of internalization via phagocytosis begins with the formation of pseudopods during and after the internalization of parasite; the parasitophorous vacuole assembles. Inside the vacuole, the promastigote transforms into amastigotes. Following the transformation, host cell lysosomes migrate and fuse with the parasitophorous vacuole. The lysosomal content is released in the vacuole. However, the parasite is not altered. Most of the amastigotes become attached to the membrane of the vacuole by their posterior portion and the amastigotes begin to divide multiple

times. The parasitophorous vacuole can occupy the entire cytoplasm of the host cell and culminates with their bursting out of the cell. Finally, the amastigotes reach the extracellular space and subsequently appear inside the monocyte or free in the bloodstream. Thus, they may be sucked by another female sandfly during the blood meal or invade other macrophages. Thus, the cycle continues between the female sandfly and the human host.



(*Chappuis, Sundar et al. 2007*)

Fig 1.8 Life cycle of *Leishmania* in the human host

Chapter 2:

REVIEW OF LITERATURE

2.1 Chemotherapy for VL treatment

2.1.1 Antimonials

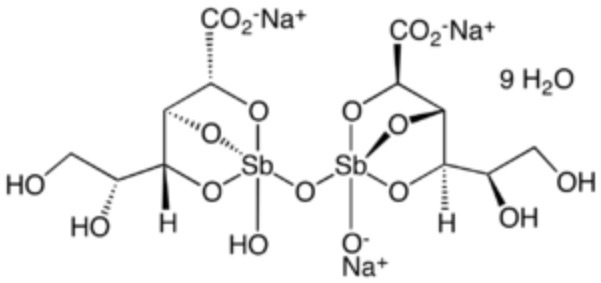


Fig 2.1 Chemical structure of sodium stibogluconate

Sodium stibogluconate, the pentavalent antimonial, is the first line of drug for the treatment of leishmaniasis. The pentavalent antimoniate (Sb^V) is the pro-drug and its trivalent form (Sb^{III}) is the active form. However, the parasite is susceptible to the pentavalent form of this drug also (*Ephros, Bitnun et al. 1999*). But it's not known if this conversion occurs in macrophage or parasite (*Shaked-Mishan, Ulrich et al. 2001*). The reduction in parasite is supported by the presence of thiol-dependent reductases (TDR) 1 enzyme that catalyzes the conversion of Sb^V to Sb^{III} using glutathione as a reductant (*Torres, Adaui et al. 2010*). Loss of this parasite reductase activity may lead to resistance. This has been supported by the finding of the loss of reductase activity in the Sb^V resistant *L. donovani* amastigotes (*Singh, Kumar et al. 2012*). The parasite was also found to contain arsenate reductase 2 (ACR2) which increases the sensitivity to Sb^V (*dos Santos Ferreira, Martins et al. 2003*). The metal reduction by host specific enzymes in bacteria and yeast (*Rosen 2002*) support the phenomenon that the reduction takes place in macrophages rather than in parasites (*Sereno, Cavaleyra et al. 1998*). The route of entrance into either the macrophages or the parasites is not yet known. But it was observed that aquaporin 1 transporter, the aquaglyceroporin, of parasite is responsible for the transport of antimonials into the amastigotes (*Gourbal, Sonuc et al. 2004*). Even the mechanism of action is unknown. But it can kill the parasite by DNA fragmentation, β-oxidation of fatty acid and adenosine diphosphate phosphorylation, inhibition of glycolysis and metabolic pathways, increase in the efflux of intracellular thiols by ATP binding cassette (ABC) transporter, multi-drug resistant protein A

(MRPA) (*Sereno, Holzmuller et al. 2001; Lee, Bertholet et al. 2002; Sudhandiran and Shaha 2003; El Fadili, Messier et al. 2005*). They are also known to inhibit trypanothione reductase enzyme which protects the parasite from host reactive oxygen and nitrogen species (*Wyllie, Cunningham et al. 2004*). As the drug is easily available over the countries in endemic regions, it is widely misused. Such misuse of this drug and loss of drug conversion by the parasites might be the driving factors for the emergence of resistance. Various factors have been found to be associated with the resistance. The loss of reductase activity in the Sb^V resistant *Leishmania* axenic amastigotes leads to the resistance *(Bolhassani, Taheri et al. 2011*). Either reduced uptake or increased efflux of the metals reduces their intracellular accumulation conferring resistance to the parasites (*Brochu, Wang et al. 2003*). Antimonial resistant parasites have been shown to overexpress heat shock protein 70 (HSP70) gene (*Brochu, Haimeur et al. 2004*). Overexpression of transporters belonging to ABC family such as MRPA and pentamidine resistant protein 1 (PRP1) are also thought to involve in the resistance (*Coelho, Beverley et al. 2003; Maltezou 2010*). Thus, the various factors involved in the emergence of resistance suggests the multifactorial mechanism (*Singh, Singh et al. 2003; Decuypere, Rijal et al. 2005; Carter, Hutchison et al. 2006; Choudhury, Zander et al. 2008*).

2.1.2 Amphotericin B (AmB)

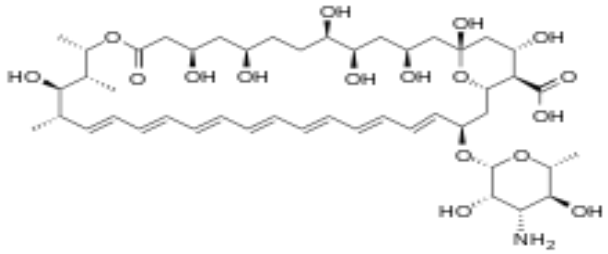


Fig 2.2 Chemical structure of amphotericin B

Amphotericin B (AmB) is a polyene antifungal drug used to treat systemic fungal infections (*Marcondes, Biondo et al. 2011*). It is preferred to antimonials for the treatment in Bihar where resistance to antimonials is common (*Bern, Adler-Moore et al. 2006*). AmB has a high affinity for ergosterol, the abundant sterol in fungal and *Leishmania* cell membranes. Though highly efficient, it was found to be toxic and imparts side effects *(Izzedine, Launay-Vacher et al. 2001;*

Laniado-Laborin and Cabrales-Vargas 2009). Three clinical formulations namely Ambiosome (liposomal AmB), Amphocil (AmB colloidal dispersion), Abelcit (AmB lipid complex) have been developed by replacing its deoxycholate with other lipids. These formulations were shown to retain antifungal activity and exhibit high efficacy with less toxicity. Though the liposomal AmB exhibits more than 95% efficacy, its exorbitant cost limits its use. AmB interacts with ergosterol of *Leishmania* and cholesterol of host macrophages for its antileishmanial activity. The formation of cholesterol-AmB complex obstructs the binding of promastigotes to the macrophage (*Paila, Saha et al. 2010*). It is also observed to induce the formation of aqueous pores leading to the osmotic changes resulting in the death of promastigotes *(Ramos, Valdivieso et al. 1996*). Even though it has high efficacy, its administration is associated with the toxicity and the emergence of parasite resistance. AmB has a damaging effect on kidney tubular cell. The reason behind this is that the formation of aqueous pores results in the increased salt and Ca^{2+} concentration and H^{+} permeability which alleviate the pH and Ca^{2+} gradient across the membrane that results in apoptosis in eukaryotic cells. Another limitation in its use is the emergence of parasite resistance which might be due to various factors. Different *in vitro* studies on resistant parasites demonstrate the lack of membranous ergosterol which is the primary target of AmB *(Mbongo, Loiseau et al. 1998*), lack of C-24 alkylated sterols in their membrane due to the inactivation of S-adenosyl methionine transferase (*Pourshafie, Morand et al. 2004*), amplification of TarII 64.4 and TarII 512.2 in *L. tarentolae* mutants (*Singh, Papadopoulou et al. 2001*). Though the clinical resistance has not been reported yet, its nonspecific mode of action at the membrane level may be a factor for its infrequent resistance. The success of AmB treatment depends on the immune status of the patient and the successive relapse might help in the emergence of resistance *(Di Giorgio, Faraut-Gambarelli et al. 1999; Lachaud, Bourgeois et al. 2009*).

2.1.3 Miltefosine

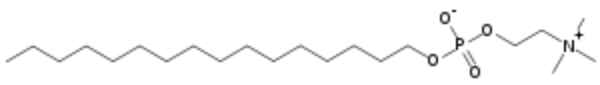


Fig 2.3 Chemical structure of miltefosine

Miltefosine, a hexadecylphosphocholine, is originally developed as an anticancerous agent *(Croft and Coombs 2003*). It was fortuitously found to be exhibiting antileishmanial activity. It is the first oral drug in chemotherapy against VL and has been considered as a major breakthrough (*Jha, Sundar et al. 1999; Sundar, Jha et al. 2006*). Its phase trials were shown promising protection against VL (*Sundar and Chatterjee 2006; Bhattacharya, Sinha et al. 2007*). Though the combinatorial effect of Miltefosine and Ambisome was effective, the side effects were of concern in its further use (*Sundar, Sinha et al. 2011*). The high efficacy of this drug is compromised by its long half-life and teratogenicity. Its long half-life of approximately 160 h could be the factor that opens the door for the emergence of resistance. The teratogenic and abortifacient properties of this drug are also the limiting factors in its usage. Intracellular accumulation of miltefosine is regulated by two transporters, LdMT and β-subunit LdROS3, a P-type ATPase, belonging to aminophospholipid translocase family *(Perez-Victoria, Gamarro et al. 2003*). The resistant *Leishmania* was found to have the reduced accumulation of miltefosine due to the decreased influx (*Pérez-Victoria, Castanys et al. 2003*). The exact mode of its antileishmanial activity remains an enigma, but it was observed to cause apoptosis in *L. donovani* (*Paris, Loiseau et al. 2004*). It decreases the parasite proliferation by reducing the lipid content in their membrane and enhancing the phosphatidylethanolamine content indicating the partial inhibition of phosphatidylethanolamine-N-methyltransferase (*Loiseau and Bories 2006*). The major cause believed for the emergence of resistance to this drug is due to the decreased intracellular drug accumulation. A single point mutation at LdMT and LdROS3, overexpression of multidrug resistant MDR1 gene which encodes for a glycoprotein might be responsible for the emergence of resistance (*Perez-Victoria, Parodi-Talice et al. 2001*). Additionally, the reduced content of unsaturated phospholipid alkyl chains in the parasites is also linked to the miltefosine resistance (*Rakotomanga, Loiseau et al. 2004*). Though the clinical resistance has not been reported yet, its improper use in endemic countries like India would lead to the incidence of resistance and spread of these resistant parasites where the prevalence of infection is significantly high. Few patients relapse after 9-12 months of successive treatment with miltefosine. However, further studies are required for the understanding whether these are relapse, or reinfection, or resistance.

2.1.4 Paromomycin

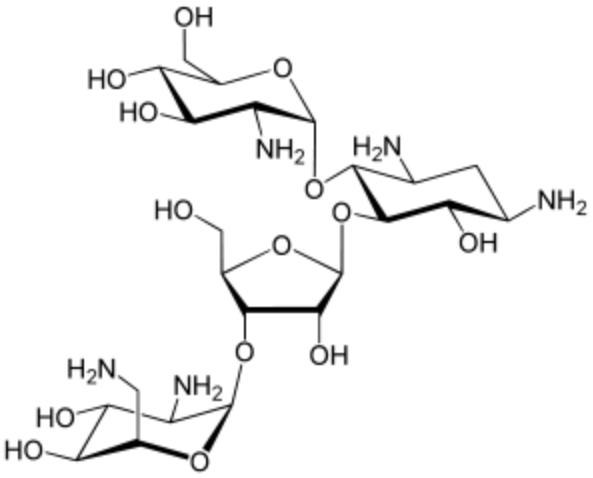


Fig 2.4 Chemical structure of Paromomycin

Paromomycin is an aminoglycosidic antibiotic with both antileishmanial and antibacterial activities. Its antileishmanial activity was discovered in 1960s. It is shown to be effective both against VL and CL. But its limited availability restricts its use in endemic regions *(Thakur, Kanyok et al. 2000; Thakur, Narayan et al. 2003*). Its mechanism remains to be unknown. But different studies reported various possibilities for its mode of action. Paromomycin, being cationic, binds to the negatively charged glycocalyx of *Leishmania* parasites and targets mitochondria (*Jhingran, Chawla et al. 2009*). It promotes the association of 50S and 30S subunits of cytoplasmic and mitochondrial ribosomes of *L. donovani* affecting their recycling and inhibits protein synthesis (*Maarouf, Lawrence et al. 1995*). It interacts with both 30S and 50S subunits but does not inhibit the association of translation initiation factor 3 (IF3) to the 30S subunit (*Hirokawa, Kaji et al. 2007*). The resistance has not been reported to this drug till now as it is limited in use, but the *in vitro* resistance has been reported in *L. donovani* and *L. tropica* (*Maarouf, Adeline et al. 1998; Jhingran, Chawla et al. 2009*). However, there is a possibility of resistance emergence against this drug due to its aminoglycosidic nature *(Singh, Kumar et al. 2012*).

2.1.5 Sitamaquine

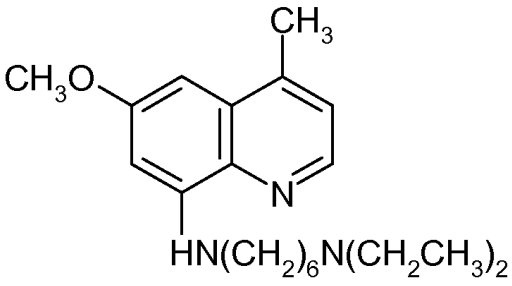


Fig 2.5 Chemical structure of sitamaquine

The chemical name of sitamaquine is 8-aminoquinoline. It is the oral drug and was developed in collaboration with GlaxoSmithKline and Walter Reed Army Institute *(Sundar and Chatterjee 2006*). In Indian phase II trial, it exhibited high efficacy against VL but inflicted few side effects such as vomiting, dyspepsia, cyanosis, nephritic syndrome, and glomerulonephritis *(Jha, Sundar et al. 2005*). Sitamaquine also showed similar efficacy in Kenyan phase II trial with the abdominal pain, headache, and kidney dysfunctioning as side effects *(Wasunna, Rashid et al. 2005*). This drug affects parasite motility, morphology, and growth *(Duenas-Romero, Loiseau et al. 2007*). The positively charged sitamaquine inserts within the biological membranes by its electrostatic interaction with the phospholipid anionic polar head groups *(Coimbra, Goncalves-da-Costa et al. 2008*). Thereafter, it accumulates in the acidocalcisomes, the cytosolic acidic compartments in *Leishmania* parasites (*Lopez-Martin, Perez-Victoria et al. 2008*). But there is no clear correlation between its action and accumulation. Though the clinical resistance against this drug has not been reported yet, *in vitro* resistance has been demonstrated in *L. donovani* against 160µM drug concentration (*Bories, Cojean et al. 2008*). A study of CL in BALB/c mice by *L. major* showed no reduction in the parasite burden with the lesion progression. This lack of efficacy restricted the clinical trials of this drug *(Garnier, Brown et al. 2006*). There is a need for further studies on this drug to understand its efficacy, mode of action, and toxicity.

2.1.6 Pentamidine

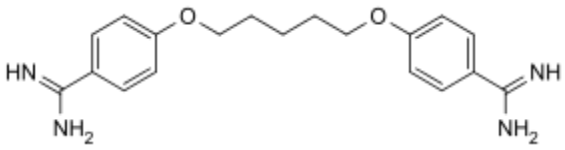


Fig 2.6 Chemical structure of pentamidine

Pentamidine, an aromatic diamine, is the second line of drug used to treat leishmaniasis. For the treatment of VL, its isothionate and methansulphonate salts are used. Initially, it was used to treat Sb^V refractory patients but later its efficacy was shown to be declining and the risk of resistance led to its closure in India. Pentamidine, in combinatorial study with allopurinol, showed high efficacy and 73% cure (*Das, Ranjan et al. 2001*). The pentamidine is thought to enter *L. donovani* through arginine and polyamine transporters but its exact mode of action is not known (*Kandpal and Tekwani 1997; Basselin, Coombs et al. 2000*). A study with pentamidine resistant *L. donovani* and *L. amazonensis* promastigotes demonstrated that the drug resistance is due to the decreased drug uptake with its increased efflux. This decreased influx of the drug might be due to the alteration in the polyamine carrier. It gets accumulated in the mitochondria and exhibits leishmanicidal activity by decreasing the mitochondrial membrane potential through increasing the efficacy of mitochondrial respiratory chain II complex inhibitors. It is also shown to inhibit mitochondrial topoisomerase II (*Basselin, Lawrence et al. 1996*). The mechanism of pentamidine resistance is not clearly understood but intracellular ABC protein PRP1 confers resistance to pentamidine in amastigotes (*Coelho, Messier et al. 2007*).

All the currently available drugs have limitations like exorbitant price, improper feasibility, low efficacy, high toxicity, side effects and the emergence of resistance. The major concern in the current chemotherapy is the gradual emergence of resistance particularly in India. The treatment of leishmaniasis has become challenging. This led to the trials of combinational therapies like LAmB (Liposomal amphotericin B)+miltefosine, LAmB+paromomycin, LAmB+antimonials, and paromomycin+antimonials (*Singh, Kumar et al. 2012*). Though the combinational drugs reduce toxicity and dosage, the resistance against drugs cannot be contradicted. As there is no

vaccine available currently against leishmaniasis, the focus of researchers has been on the vaccine development using the available candidate leishmanial antigens or immunogens.

2.2 Vaccines against leishmaniasis

Leishmania exhibits significant antigenic diversity which forestalls the vaccine development against VL. This necessitates the gain of knowledge of such antigenic diversity in *Leishmania* (*Kumari, Kumar et al. 2008*). In this regard, various attempts have been made by several researchers to identify the potential antigens that can be targeted as a suitable vaccine candidate.

The attempt of vaccine development was initially made in case of CL by using Leishmanization (LZ) in Western and South-Western Asia. LZ is the inoculation of virulent parasite from the exudates of cutaneous lesions in to an uninfected individual. It was shown to provide an active immunity through the formation of self-healing lesions *(Bray, Modabber et al. 2000*). The killed vaccination trial in Brazil showed a promising protection role inducing IFN-γ and reducing IL-4 levels which represents the Th1-type immune response (*Pessoa 1941*). Killed *Leishmania* in combination with antimonial therapy enhanced cure rates and reduced replase incidence *(Musa, Khalil et al. 2008*). The whole killed parasites were recommended for therapeutic and prophylactic purposes (*Basyoni 2012*).

A number of antigens have been evaluated with varied success rates in various animal models. Hence, the selection of suitable vaccine candidates becomes a difficult task in case of leishmaniasis (*Basyoni 2012*). The features such as genetic variation and polymorphism in *Leishmania (Kumar and Engwerda 2014*) insist for the alternative approaches for generation of better vaccine.

Several species and stage specific leishmanial molecules have been used as vaccine candidates in the form of recombinant proteins. A recombinant protein A2 with saponin provided protection with significantly elevated levels of IFN-γ and low IL-10 levels against *L. chagasi* infection in dogs (*Fernandes, Costa et al. 2008*). Kumari *et al.* evaluated the recombinant proteins such as elongation factor-2 (eIF-2), triose phosphate isomerase (TPI), protein disulfide isomerase (PDI), aldolase, enolase, P45, and trypanothione reductase (TPR) from the soluble fractions of promastigotes *(Kumari, Samant et al. 2008; Kumari, Samant et al. 2008*). The study on

immunogenicity of LeIF-2, TPI and PDI of *L. donovani* was found to increase IFN-γ, IL-12, TNF-α, and IgG2 levels (*Kushawaha, Gupta et al. 2011; Kushawaha, Gupta et al. 2012; Kushawaha, Gupta et al. 2012*). Other proteins p45, enolase, and aldolase were also found to be the potential vaccine candidates with an increased iNOS, IFN-γ, TNF-α, IL-12 and decreased TGF-β and IL-4 *(Gupta, Kushawaha et al. 2012; Gupta, Kumar et al. 2014*). rLdTPR+BCG vaccination against *L. donovani* challenge in hamsters was protective with increased inducible NO synthase (iNOS), IFN-γ, IL-12, and TNF-α levels and decreased IL-4, IL-10, and TGF-β levels (*Khare, Jaiswal et al. 2014*).

The multicomponent or polyprotein preparations, for example, Q protein, Leish-111f, Leish-110f, KSAC, etc. have been used to evaluate their protective efficacy against experimental VL. Among these, Q protein along with BCG showed 90% protection in dogs whereas Q protein with CpG-ODN motifs induced long-lasting IgG response in mice *(Molano, Alonso et al. 2003; Parody, Soto et al. 2004*). Another polyprotein named Leish-111f was ineffective against *L. infantum* challenge (*Gradoni, Manzillo et al. 2005*) but, when combined with adjuvant MPLA-stable emulsion (MPL-SE), provided significant protection in mice, hamsters *(Coler, Goto et al. 2007*), and dogs (*Trigo, Abbehusen et al. 2010*) with reduced parasitemia and increase in Th1 cytokine levels. Leish-110f, a formulation of Leish-111f, along with natural MPL-SE or synthetic (EM005) TLR-4 agonists generated good humoral and cellular responses *(Bertholet, Goto et al. 2009*). Another polyprotein named KSAC with MPL adjuvant was immunogenic and provided significant protection against *L. infantum* challenge in mice (*Goto, Bhatia et al. 2011*).

DNA vaccines are prepared by the cloning of genes encoding the target protein into the mammalian expression vector. The major limitations of these vaccines are stability, safety, long-term protection, administration, and cost effectiveness. Molecules evaluated for this approach are A2, P36LACK, PapLe22, ORFF, KMP-11 proteophosphoglycan (PPG), etc. in different models. A2 (*Ghosh, Zhang et al. 2001*), ORFF (*Sukumaran, Tewary et al. 2003*), truncated 24-kDa LACK antigen (*Melby, Yang et al. 2001*) in BALB/c mice against VL, PapLe22 in golden hamster (*Fragaki, Suffia et al. 2001*) conferred significant protection. In another trial of DNA vaccination, NH36 was found to provide significant protection against *L. chagasi* in BALB/c mice (*Aguilar-Be, da Silva Zardo et al. 2005*). Other various studies demonstrated the use of

pClneo-LACK in BALB/c mice (*de Oliveira Gomes, Pinto et al. 2007*), KMP-11 in hamsters (*Basu, Bhaumik et al. 2005*) and BALB/c mice (*Bhaumik, Basu et al. 2009*), N-terminal domain of PPG gene in the golden hamsters (*Samant, Gupta et al. 2009*), hemoglobin receptor (HbR)-DNA in BALB/c mice (*Guha, Gupta et al. 2013*) for the DNA vaccination. Enzymes such as pVAXγGCS (gamma-glutamyl cysteine synthetase) (*Carter, Henriquez et al. 2007*) and UBQ-ORFF (*Sharma and Madhubala 2009*) were also shown to confer protective response.

Heterologous DNA-prime protein-boost (HPB) is another strategy used for vaccination and the VL antigens used in this approach were ORFF, cysteine proteinases, GP63, etc. Immunization with DNA-LACK primer/rVV-LACK boost against *L. infantum* challenge showed 60% protection in dogs (*Ramiro, Zárate et al. 2003*) as well as in murine models *(Dondji, Pérez-Jimenez et al. 2005; Tewary, Jain et al. 2005*). The combination of cysteine proteinases DNA/protein with ORFF DNA/protein *(Rafati, Nakhaee et al. 2005; Rafati, Zahedifard et al. 2006*) and GP63 membrane protein in BALB/c mice (*Mazumder, Maji et al. 2011*) conferred robust immune responses with long-lasting protection against *L. donovani* challenge.

During the blood feeding, the vector sandfly delivers its salivary proteins into the host during natural transmission of pathogen and act as immunomodulatory molecules for the host (*Basyoni 2012*). Several salivary proteins such as PpSP15, maxadilan, LJM17, LJM19, and LJM143 were found to be potent immunogens that induce lymphocytic infiltration enhancing IFN-γ and IL-12 (Gomes, Teixeira et al. 2008; Collin, Gomes et al. 2009). Several proteins were found to be demonstrated as potential vaccine candidates; a very few have reached to the clinical trials (*Duthie and Reed 2014*) due to the variations in immunogenicity and genetic variation in host and pathogen (*Kumar and Engwerda 2014*). Hence, there is a pressing need for the development of a potential drug. One of the possibilities of developing the potential drug is the rational redesigning of the currently available effective drugs. Although the emergence of resistance in the parasite is the only limitation posed by the miltefosine drug, it is the only effective drug among the currently available drugs. Hence, understanding the mechanism of resistance in the parasite against miltefosine could help in the redesigning of presently available miltefosine drug.

2.3 Background study:

The miltefosine compound was synthesized by two different research groups in UK and Germany (*Smorenburg, Seynaeve et al. 2000*). Though this compound was shown to be active against trypanosomatid parasites, it was not of much focus earlier. But the *in vitro* anti-leishmanial demonstrations on *Leishmania*, high bioavailability in pre-clinical studies and its easy oral administration encouraged the researchers to evaluate its antileishmanial activity in mouse models (*Kuhlencord, Maniera et al. 1992*). The successful phase II studies against human VL in India (*Sundar, Rosenkaimer et al. 1998*) resulted in the collaboration between ASTA Medica, the WHO Special Program for Research and Training in Tropical Diseases, and the Government of India (*Engel 2002*). Later, in 2002, miltefosine has been approved as the first and the only oral drug for the treatment of VL. Miltefosine is chemically called as hexadecyl 2-(trimethylazaniumyl) ethyl phosphate and also as hexadecylphosphocholine with the empirical formula $C_{21}H_{46}NO_4P$ and molecular weight of 407.57 g/mol. It is an amphiphilic and zwitterionic compound due to the positively charged amino group and negatively charged phosphoryl group. The crystalline compound is a white hygroscopic powder and is readily soluble in aqueous and organic solvents.

2.3.1 Uptake of miltefosine

The accumulation of short-chain phospholipids and its derivatives such as miltefosine can be divided into three steps.

(i) Binding of miltefosine to the outer layer of the plasma membrane:

Albumin serves as a reservoir for the miltefosine. Hence, the drug binds to albumin under normal culture conditions. As the drug is water soluble, it is also able to bind individually to the monolayers (*Rakotomanga, Saint-Pierre-Chazalet et al. 2005*).

(ii) Internalization of the miltefosine:

Usually, the phospholipid molecules diffuse rapidly within a lipid monolayer. Although, their flip-flop movement requires days and is generally slow. The internalization of the drug is possible via two mechanisms.

(a) Endocytic pathway: The miltefosine monomers are internalized as the members of the endocytic vesicles. Such endocytic activity is highly pronounced in *Leishmania* parasites at the flagellar pocket (*McConville, Mullin et al. 2002*).

(b) Non-endocytic pathway or flippase activity: This is the most contributing pathway for the uptake of miltefosine (*Pérez-Victoria, Castanys et al. 2003*). This type of uptake depends on the complex of LdMT transporter and its beta subunit LdRos3.

(iii) Intracellular targeting and metabolism:

The intracellular distribution of miltefosine is unknown hitherto. But as the drug is water soluble, it is thought to equilibrate within the membranes of internal organelles. Though the internalization of the drug is very high, its metabolism is very slow or negligible (*Pérez-Victoria, Castanys et al. 2003*).

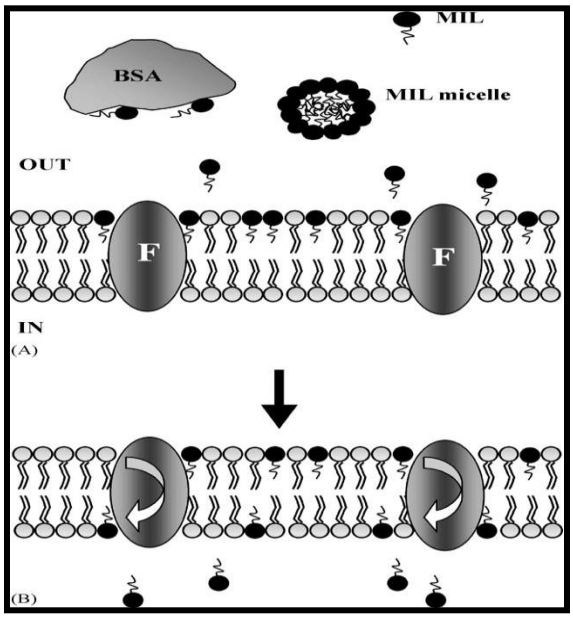


Fig 2.7 Binding and uptake of miltefosine in *Leishmania* parasites. (A) Binding of the drug to the outer leaflet of the plasma membrane. Miltefosine is recruited by bovine serum albumin (BSA), which acts as a reservoir for the drug. (B) The fraction bound to cell membranes is internalized through a flippase protein (F) machinery present at the lipid bilayer. This translocation machinery includes, at least, two proteins at the plasma membrane: the miltefosine transporter LdMT and its beta subunit LdRos3.

(*Pérez-Victoria, Sánchez-Cañete et al. 2006*)

2.3.2 Antileishmanial activity of miltefosine

The antileishmanial activity was first reported by Croft et al (*Croft, Neal et al. 1987*). Though the exact antileishmanial mechanism of miltefosine is not known, similar modes of antileishmanial and anticancerous actions were observed. But several studies reported various hypotheses for its antileishmanial mechanism of action as depicted in the following figure. These multitudes of contradictory mechanisms suggest that miltefosine has more than one molecular site of action.

The probable targets of miltefosine in *Leishmania* parasite are fatty acid and sterol metabolism (*Rakotomanga, Saint-Pierre-Chazalet et al. 2005*). Miltefosine was shown to exert its effect on the parasite's phospholipid metabolism and membrane composition with a decrease in phosphatidylcholine and an increase in phosphatidylethanolamine *(Rakotomanga, Blanc et al. 2007*). This finding correlates with the miltefosine-mediated inhibition of the entry of exogenous choline into the parasite (*Zufferey and Mamoun 2002*). Miltefosine induces apoptotic like death in *Leishmania* promastigotes resulting in nuclear condensation and oligonucleosomal DNA fragmentation (*Das, Mukherjee et al. 2001*). In addition, miltefosine was also observed to cause mitochondrial dysfunction in the parasites by exerting a decrease in mitochondrial membrane potential (*Santa-Rita, Henriques-Pons et al. 2004*) and inhibiting cytochrome-c oxidase *(Luque-Ortega and Rivas 2007*).

Besides its direct action on parasites, miltefosine is also known to exert immunomodulatory effects on *Leishmania* infected macrophages. It enhances IFN-γ response and promotes the immune response towards Th1 type by inducing the IL-12 (*Wadhone, Maiti et al. 2009*). But the immunostimulatory action of miltefosine is also enveloped by controversy. A report stated that miltefosine neither upregulated major histocompatibility complex II (MHC-II) nor altered the release of cytokines IL-10, IL-12 or TNF-α (*Griewank, Gazeau et al. 2010*).

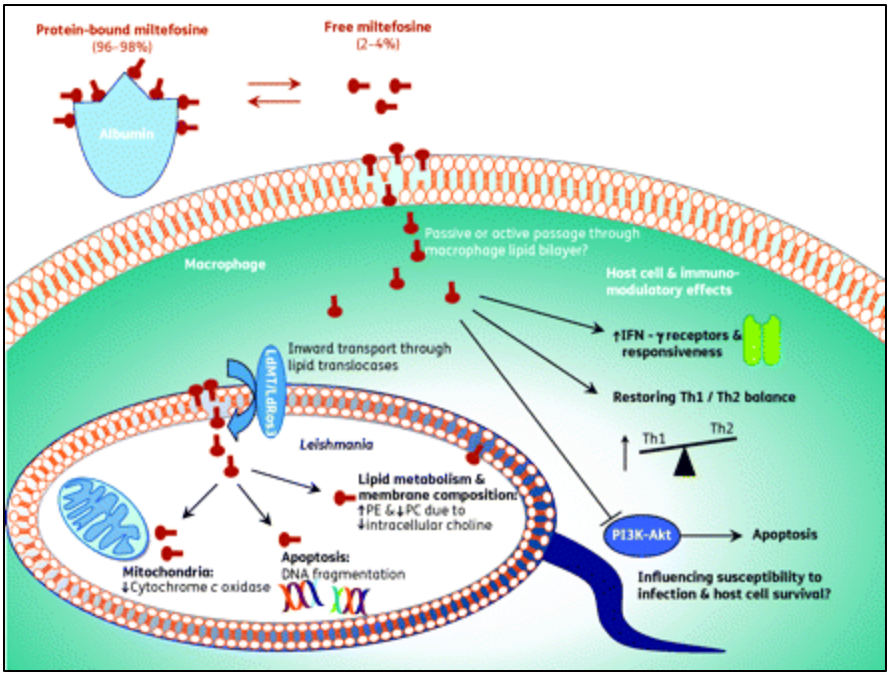


Fig 2.8 Antileishmanial mechanism of action of miltefosine. The various proposed mechanisms of action of miltefosine against the intracellular *Leishmania* parasite and the macrophage host cell during leishmaniasis infection. PC, phosphatidylcholine

(*Dorlo, Balasegaram et al. 2012*)

2.3.3 Effective dose of miltefosine

Miltefosine has been registered in India, Germany and Colombia. The dosage recommended for the treatment is 100-150 mg/day or 2.5 mg/kg body weight for 28 days. It was observed to induce 95% clinical and parasitological cure for VL and 91% for CL. The drug miltefosine is also effective in Indian children *(Sundar, Jha et al. 2003; Bhattacharya, Jha et al. 2004*). The pharmacokinetics and side effects of miltefosine were found to be similar in both the children and the adults.

2.4 Aim of the study

Miltefosine has a long half-life in human plasma ranging between 150-200 h. The drug levels in plasma are dose proportional and urine excretion is also negligible as cytochrome P450 is unable

to metabolize the drug. To attain more than 90% clearance, about four half-lives i.e. 25-33 days are required indicating the existence of subtherapeutic levels for some weeks even after the standard course of treatment. Such remnants of drug for longer periods in the human plasma and its exposure to the parasite aids in the emergence of resistance in the parasite against the drug (*Bryceson 2001*). Several studies carried out on the experimental *L. donovani* strains resistant to miltefosine have reported various reasons. The miltefosine resistance could be attributed to defective inward translocation of miltefosine (*Pérez-Victoria, Castanys et al. 2003*). This has been related to the single nucleotide polymorphism (SNP) L832F in drug transporter Ldmt gene *(Cojean, Houzé et al. 2012*). The overexpression of ABC transporter P-glycoprotein MDR1 *(Perez-Victoria, Parodi-Talice et al. 2001*) could also contribute for the miltefosine resistance as it is involved in the efflux of the drug. Till date, the only explained mechanisms for the miltefosine resistance are either the decreased drug uptake or increased drug efflux. Hence, further investigation has to be carried out for the exploration of resistance mechanism against the drug for the rational redesigning of the efficient drug that can overcome the resistance mechanism. Therefore, we hypothesize that the differentially regulated proteins in the sensitive and resistance mechanism could provide hints to the resistance mechanism.

Based on this, our study was divided into the following objectives.

1. Comparative proteomics of miltefosine sensitive and resistant parasites
   - **a.** Validation of the clinical isolate, BHU875 as a miltefosine resistant strain in vitro and ex vivo as compared to sensitive isolate, DD8.
   - **b.** Analysis of differentially regulated proteins in the promastigote stage of BHU875 as compared to DD8.
2. Knockdown of FesodA gene in the miltefosine sensitive and resistant parasites to understand its role against miltefosine drug.
3. LC-MS/MS analysis of host macrophage proteins during the infection with miltefosine sensitive and resistant parasites.

Chapter 3:

MATERIALS AND METHODS

3.1 Parasites, Cell lines, and Animals

- *L. donovani* strain DD8 (MH0M/IN/80/DD8) was procured from ATCC, USA. The miltefosine resistant *L. donovani* clinical isolate BHU875 was kindly provided by Prof. Shyam Sundar, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.
- J774A.1 murine macrophages were obtained from National Centre for Cell Science, Pune, India.
- Inbred female BALB/c mice of about 4-6 weeks old used for the study were purchased from National Centre for Laboratory Animal Sciences, Hyderabad, India. All the mice were maintained at Animal House, School of Life Sciences, University of Hyderabad, India.

3.2 General chemicals and reagents

All the biochemicals and immunochemicals used in the study have been listed hereunder.

M199 and DMEM media, Penicillin/Streptomycin antibiotic mixture, $MgCl_2$, $CaCl_2$, D-glucose, FBS, Sodium bicarbonate ($NaHCO_3$), IPTG, Anti-mouse IgG ALP conjugate produced in goat, Betaine, Imidazole, BCIP/NBT solution premixed, Propidium iodide (PI), 6-biopterin, Protease inhibitor cocktail, CHAPS, 4% Formalin, Freund's incomplete adjuvant, and Freund's complete adjuvant were procured from Sigma-Aldrich, USA. Miltefosine was purchased from Cayman Chemicals, USA. PBS was acquired from Gibco. Griess assay kit was obtained from Invitrogen. Urea, Thiourea, DTT, Skimmed milk powder, Tris buffer, Glycine, SDS, Triton X-100, Glycerol, Hydroxyurea, NaCl, Tween-20, Acrylamide, Kanamycin sulphate, Agar Agar, Peptone, Yeast extract powder, Giemsa stain, Ammonium bicarbonate, MTT, Sodium lauroyl sarcosine, Proteinase K, DNase I, RNase, Amido Black, G418 sulphate, Acetonitrile, TFA, and Trypan blue were procured from Hi-Media, Mumbai, India. Orthophosphoric acid, DreamTaq Polymerase, Phusion High-Fidelity DNA polymerase, Protein molecular weight markers, DNA ladders, T4 DNA ligase, Restriction enzymes, Enzyme Buffers, SnakeSkin® dialysis tubing (3.5K MWCO), and Electroporation cuvettes were procured from Thermo Fisher Scientific Inc. Ethidium bromide, Ponceau S, Coomassie Blue G-250, Coomassie Blue R-250, Immersion oil for microscopy, β-mercaptoethanol (βME), PMSF, EDTA, TEMED, BSA, Bis-acrylamide, Tris-saturated phenol, Isopropyl alcohol, Isobutanol, Glacial acetic acid, Ammonium sulphate,

Disodium hydrogen orthophosphate anhydrous, Sodium dihydrogen orthophosphate, Sodium hydroxide (NaOH), Bromophenol blue, HEPES buffer, KCl, and Trypsin were procured from SRL, India. Plasmid purification kit, PCR and Gel clean up kit, and RNA isolation kit were purchased from Macherey-Nagel. DMSO and Chloroform were procured from Merck (India). PCR primers were obtained from Integrated DNA Technologies, USA. HPLC-grade Methanol and Paraformaldehyde were procured from Qualigens. SeaKem® LE Agarose for gel electrophoresis was procured from Lonza, USA. pET28a expression plasmid was procured from Novagen, USA. pEGFP-C1 mammalian expression vector was procured from Invitrogen, USA. Ni Sepharose 6 Fast Flow, IPG strips (pH 3-10), and IPG buffer were procured from GE Healthcare. The Nitrocellulose membrane was purchased from Pall Life Sciences. ECL Western Blotting detection reagent was procured from Amersham Biosciences, India. SYBR Green master mix and cDNA synthesis kit were purchased from Takara. H_2DCFDA and Anti-His antibody were procured from Life Technologies, USA. Cell culture flasks, dishes, serological pipettes were obtained from Corning, India.

3.3. Culture media

3.3.1 Growth media for L. donovani promastigotes

M199 powder (10.6 g) was dissolved in 800 ml of autoclaved double distilled water supplemented with 0.35 g of $NaHCO_3$ and the pH was adjusted to 7.4. The final volume was adjusted to 1 L by adding autoclaved ddH_2O that serves as an incomplete medium. About 15% heat inactivated FBS and 1X penicillin/streptomycin antibiotic mixture were added to the incomplete medium to make it a complete medium. Thus resulting complete medium was filter sterilized through a 0.22 μm membrane filter and stored at 4^oC prior to use.

3.3.2 Growth media for culturing macrophages

RPMI1640 (16.4 g) was dissolved in 800 ml of double autoclaved distilled water supplemented with 2 g of $NaHCO_3$, and the pH of this solution was adjusted to 7.5. The final volume was adjusted to 1 L with ddH_2O that serves as an incomplete medium. About 10% heat inactivated FBS and 1X penicillin/streptomycin antibiotic mixture were added to the incomplete medium to make it a complete medium. Thus resulting complete medium was filter sterilized through a 0.22 μm membrane filter and stored at 4^oC prior to use.

3.3.3 Growth media for bacterial culture

3.3.3.1 Luria-Bertani (LB) broth

Peptone (10 g), Yeast extract (5 g), and NaCl (5 g) were dissolved in 800 ml of double distilled water. The pH was adjusted with 10 N NaOH until it reaches 7.4 after which the volume was adjusted to 1000 ml. Later, the medium was sterilized by autoclaving for 15 min at 15 lbs pressure.

3.3.3.2 LB agar plate

For the solidification of LB broth, 15 g of agar agar was added to 1 L of LB broth and sterilized by autoclaving. The media temperature was brought down to 50^{o}C at room temperature (RT) and at this point, 50 μg/ml kanamycin was added to the media. Then, about 25-30 ml media was poured into each 100 mm petri plate. Such poured plates were dried under the laminar air flow (*Sambrook and Maniatis 1989*).

3.4 Culturing of *Leishmania*

3.4.1 In vitro culturing

L. donovani promastigotes were grown in M199 supplemented with 15% FBS and 1X penicillin-streptomycin antibiotic mixture and maintained at 25^{o}C in a BOD incubator. The growth cycle of the parasites usually takes 7 days. Sub-culturing of the parasites is generally carried out at the log phase. Late log phase (or stationary phase) parasites were harvested at 3500 rpm for 20 min. The parasite number was determined by Neubauer's chamber by fixing and immobilizing them with 4% paraformaldehyde.

3.4.2 In vivo culturing

The virulence of both the strains (DD8 and BHU875) was maintained by passaging in BALB/c mice. Briefly, 1x10^{8} virulent promastigotes in PBS were injected intravenously in mice. After one month of post-infection (p.i.), the spleens were harvested from the mice, homogenized under sterile conditions, and maintained in complete M199 medium for about 48-72 h at 25°C. These cultures were then observed under the microscope for the transformed promastigotes. Post confirmation of parasite transformation, they were centrifuged at 100 x g for 10 min at 4°C to remove splenic debris. Thereafter, the virulent promastigotes were harvested at 800 x g for 15 min at 4°C and resuspended in PBS at a ratio of 10^{8} parasites per ml. These virulent promastigotes were again injected intravenously in mice to maintain their virulence.

3.5 Culturing of J774A.1 murine macrophages

The J774A.1 murine macrophages were grown at 37°C in DMEM medium supplemented with 10% FBS and maintained in a CO_2 incubator injected with 5% CO_2. The macrophages were seeded into tissue culture plates at the required density and incubated for 24 h prior to the infection studies.

3.6 Survival and infectivity assays in murine macrophages

3.6.1 Assessment of fluorescence intensity in murine macrophages

The fluorescence intensity was assessed in J774A.1 murine macrophages by confocal microscopy. The macrophages ($1x10^6$) in DMEM medium were plated into 60 mm culture discs and allowed them to adhere overnight. The coverslips were also placed in the culture discs. Then the non-adherent cells were washed off and the cells were supplemented with fresh DMEM. These cells were further infected with the wild-type and knockdown parasites (of both DD8 and BHU875) for about 6 h and 72 h. The coverslips were taken after the required time points. They were placed onto the glass slides, Vectashield with DAPI was applied, the coverslips were sealed, and the GFP fluorescence was observed under confocal microscope.

3.6.2 Amastigote counting

The amastigote number was counted in the infected macrophages by Giemsa staining. Briefly, $1x10^6$ J774A.1 murine macrophages in DMEM were plated into 60 mm culture discs and incubated overnight for their adherence. The coverslips were also placed in the culture discs. After the overnight incubation, the non-adherent cells were washed off and fresh DMEM was added. Subsequently, the cells were infected with wild-type and knockdown parasites (of both DD8 and BHU875) for about 6 h and 72 h. The coverslips with adherent cells were taken at the required time points and placed onto the glass slides. The coverslips were allowed to dry and flooded with methanol. These were then stained with Giemsa stain for 5 min and washed with ddH_2O. Later, the intracellular amastigotes were counted per 100 macrophages and represented in a bar graph.

3.7 Parasite staining

The parasite was stained using Giemsa stain. Briefly, the parasites were smeared onto a slide and allowed to dry. Then the parasites were fixed with methanol and subsequently stained with

Giemsa stain for about 15-20 min. Later, the slide was flooded with ddH_2O and allowed to dry which then was observed under the microscope.

3.8 Determination of miltefosine IC_{50} with sensitive and resistant parasites

The cytotoxic effect, of miltefosine against sensitive (DD8) and resistant (BHU875) strains, was determined by MTT assay. Briefly, exponentially growing parasites ($1x10^6$ per ml of M199 medium in each well) were inoculated in 96 well plates. These parasites were treated with different concentrations of miltefosine. The concentrations of miltefosine used for treating DD8 parasites were 0 μM, 2.5 μM, 5 μM, 10 μM, 20 μM, and 40 μM. Whereas in case of BHU875 parasites, the concentrations are 0 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, and 100 μM. After 72 h of incubation at 25^{o}C, the plates were spun at 3000 rpm for 15 min. The media was aspirated and substituted with fresh media. Thereafter, 0.5 mg/ml MTT was added and incubated for 4 h at 37^{o}C with 5% CO_2. Later, the plate was centrifuged at 1000 g for 5 min to pellet down the formed formazan crystals, and the media was discarded. These purple crystals were dissolved in 100 μl DMSO, and the color absorbance was read at 540 nm. The viability percentage was calculated was deduced from the formula: Absorbance of treated/Absorbance of control X 100. The IC_{50} was deduced from the graph.

3.9 Flow cytometric analysis of parasite cell cycle

The parasite cell cycle was analyzed using PI dye exclusion test. Briefly, the exponentially growing ($2x10^7$ per ml) *L. donovani* parasites (DD8 and BHU875) were synchronized to G1 phase by 5 mM hydroxyurea treatment for about 12-16 h. After synchronization, the parasites were washed with PBS once at 2000 x g for 10 min to remove hydroxyurea and resuspended in M199, and treated with 40 μM miltefosine. These parasites were incubated for different time points viz. 6 h, 24 h, and 48 h. An unstained and hydroxyurea controls (i.e. harvested immediately after hydroxyurea treatment) were also collected. Subsequently, thus treated parasites were collected at 5000 rpm for 5 min and washed once with PBS. Then the parasites were fixed in ice-cold 70% ethanol in PBS at -20^{o}C for about 24 h. Thus fixed parasites were centrifuged, ethanol was discarded, the parasites were washed once with PBS, and were resuspended in 500 μl PBS. To this, 40 μg/ml RNase and 25 μg/ml PI were added and incubated for about 30-45 min at 25^{o}C in the dark. The fluorescence intensity of thus processed parasites was analyzed in BD LSRFortessa^{TM} using FACSDiva software.

3.10 Measurement of reactive oxygen species (ROS)

To assess differential ROS production in DD8 and BHU875 *L. donovani* parasites, the cell permeable dye H_2DCFDA was used. For this, both the strains at a concentration of $1x10^6$ parasites per ml were treated with 40 μM miltefosine for about 24 h. In addition, untreated and unstained control parasites were also maintained for 24 h. After the treatment, the parasites were collected and centrifuged at 5000 rpm for 5 min and washed once with PBS. Then the parasites were incubated with 10 μM H_2DCFDA in PBS for 15 min in dark at RT. The green fluorescence of H_2DCFDA was measured using flow cytometry (BD LSRFortessa^{TM} using FACSDiva software) as mean fluorescence intensity (MFI), and MFIs were represented as bar graph.

3.11 Nitric oxide assay

J774A.1 macrophages ($1x10^6$) in DMEM were plated in 60 mm culture dishes and incubated for 24 h at 37^{o}C with 5% CO_2 to allow them to adhere. Then the non-adherent macrophages were washed off, and fresh media was added. The adherent macrophages were infected with DD8 and BHU875 ($1x10^7$ in each case and also in triplicates) for 6 h. After 6 h treatment, the unbound parasites were washed off and replaced with fresh media supplemented with 1 μM miltefosine for about 72 h at 37^{o}C. The supernatants were then collected from all the experimental conditions (i.e. (i) uninfected, (ii) DD8 infected, (iii) DD8 infected and treated, (iv) BHU875 infected, and (v) BHU875 infected and treated). The collected supernatants were used for nitrite estimation with Griess reagent kit (*Stuehr and Nathan 1989*). Briefly, 100 μl of the collected supernatant was mixed with 100 μl Griess reagent (1% Sulfanilamide, 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 2.5% H_3PO_4). This mixture was incubated for 30 min at RT, and the resulting color absorbance was read at 540 nm (*Green, Wagner et al. 1982*). $NaNO_2$ is the standard used for the determination of nitrite concentration.

3.12 Quantitative Real-time PCR

3.12.1 RNA isolation

RNA isolation was executed by using Nucleospin RNA extraction kit according to the manufacturer's instructions. Total RNA was extracted from $5x10^6$ J774A.1 macrophages and $2x10^8$ DD8 and BHU875 parasites. RNA concentration was estimated using Nanodrop.

3.12.2 cDNA preparation

The total RNA (900 ng) was used for cDNA synthesis by reverse transcription by using First

Strand cDNA synthesis kit and the samples were stored at -80^{o}C till further use.

3.12.3 Program and data analysis

The real time primers used for 3 mouse genes and 10 parasite genes are listed in the tables below.

Table 3.1 List of the primers for mouse genes

S.No	Mouse gene	Primer	5'-Sequence-3'
1	GAPDH	Forward primer	CAAGGCTGTGGGCAAGGTCA
		Reverse primer	AGGTGGAAGAGTGGGAGTTGCTG
2	IFN-γ	Forward primer	TCAAGTGGCATAGATGTGGAAGAA
		Reverse primer	TGGCTCTGCAGGATTTTCATG
3	IL-10	Forward primer	GGTTGCCAAGCCTTATCGGA
		Reverse primer	ACCTGCTCCACTGCCTTGCT

Table 3.2 List of the primers for *L. donovani* parasite genes

S.No	*L. donovani* gene	Primer	5'-Sequence-3'
1	Actin	Forward primer	TGGCACCATACCTTCTACAACGAG
		Reverse primer	CGTCATCTTCTCACGGTTCTGC
2	Enolase	Forward primer	ATGGCGATCAGCAAGGC
		Reverse primer	ACGTTGAAGCACGGCACA
3	Ubiquitin 16S	Forward primer	ATCCAGGACAAGGAGGGCAT
		Reverse primer	CAGGTGCAGCGTGGACT
4	Tryparedoxin	Forward primer	CACTTGGGGGATGTGTTGA
		Reverse primer	ACCAGCTTGCCGAGAAGT
5	Iron superoxide dismutase	Forward primer	ACGCTCGGCTTCAACTA
		Reverse primer	CGCTGTGGTGCTTCTTGT
6	Phosphomannomutase	Forward primer	AAGGCTCTTGGGAACGAGAA
		Reverse primer	GGGAGAGACGTTGAACATAC
7	Hypothetical protein	Forward primer	CGAACAAGCGCAAGTTCG
		Reverse primer	TCGGCAATTGCAGTACCG

8	3-Ketoacyl coA thiolase like protein	Forward primer	GGCAGCAAGCTCTTCATTG
		Reverse primer	TGTCCTCCTTGCCGTTGA
9	Metallopeptidase 32	Forward primer	CCAAGACTATGATGCCCT
		Reverse primer	TTGGCCCGCCTCTGCTT
10	Metallopeptidase 16	Forward primer	CTACGAGCCGATTGCCTA
		Reverse primer	CCAAGGAAGCCGCACT

The synthesized cDNAs for the above genes were used to perform real-time PCR in Eppendorf Realplex4 real-time PCR system for the genes. GAPDH was used as a control gene for both IFN-γ and IL-10 whereas the actin for parasite genes. Briefly, the reaction mixture of quantitative real-time PCR was prepared using SYBR Green PCR master mix. The 20 µl mixture contained 1X SYBR Green, 10 pmol of each primer, and 1 µl of cDNA and the rest of the volume was made up with Milli-Q water. The reaction conditions maintained for the real-time PCR were: 2 min initial activation at 95^{o}C, 15 sec denaturation at 95^{o}C, and 30 sec annealing at 60^{o}C. This is followed by melt curve analysis, detection of the dequenched probe, calculation of threshold cycles (Ct values), and further analysis by Sequence Detection software. Relative changes in the levels of cytokines IFN-γ and IL-10 were normalized to GAPDH and the relative changes of the parasite genes' levels were normalized to actin. Later, the relative changes were quantified by $\Delta 2^{-ddCt}$ method. Thus, the fold changes were calculated and expressed as mean ± SD from the three independent experiments.

3.13 Two-dimensional electrophoresis of *L. donovani* (DD8 and BHU875) promastigotes

3.13.1 Sample preparation for 2D gel electrophoresis

Both the DD8 and BHU875 parasites were grown to stationary phase and harvested at 5000 rpm, 4^{o}C for 20 min. The parasite pellets were washed twice with 1X ice-cold PBS, pH 7.4. The resultant pellets were resuspended in lysis buffer (20 mM Tris-HCl pH 7.4, 40 mM NaCl, and 1X Protease inhibitor cocktail). These samples were subjected to sonication and centrifuged for about 10 min at 2000 rpm at 4^{o}C to remove debris. The supernatants were subjected to TCA-acetone precipitation. The resultant protein pellets were resuspended in 2D solubilization buffer (8 M urea, 2 M thiourea, 4% CHAPS, 1% Triton X-100, 0.04 M Tris-HCl pH 7.4). 1%

ampholytes (IPG buffer) and 100 mM DTT were added just prior to the experiment.

3.13.2 2D gel electrophoresis

The solubilized protein sample was applied onto 18 cm IPG strips (pH 3-10) and subjected to rehydration for about 16 h and then to IEF (500 V for 1 h; 1000 V for 5 h; 10,000 V for 8 h; 10,000-70,000 V for 1 h; 500 V for 10 h). These IPG strips were placed gently onto a 12.5% SDS-PAGE, and the 2D electrophoresis was run until the bromophenol blue dye reached the bottom of the gel (*O'Farrell 1975*). The gels were stained using colloidal coomassie staining protocol and destained with water until the spots were visible. The gels were scanned and analyzed using IMP software. The differentially expressed proteins were selected and identified using MALDI TOF-TOF analysis.

3.13.3 In-gel digestion

The differentially expressed protein spots were excised, washed with desalted water followed by 50% v/v AcCN in 25 mM $NaHCO_3$ pH 8.0, shrunk by dehydration in acetonitrile, and vacuum dried. Gel pieces were reswollen in 10-20 µl of digestion buffer containing 10 µg/ml trypsin. Later, 25 µl of 50 mM $NaHCO_3$ was added to keep the gel pieces wet during tryptic cleavage (37^oC, overnight). To extract the peptides, 50% AcCN:0.3% TFA solution was added, and the samples were incubated for 15 min and vortexed. The separated liquid was vacuum dried, and the peptides were redissolved in 10 µl 0.1% TFA. This peptide solution was mixed with a double volume of matrix, α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 50% AcCN, 0.1% TFA and spotted onto a MALDI sample plate and subjected to MALDI TOF-TOF analysis by using the mass spectrometer from Bruker Daltonics.

3.14 LC-MS/MS analysis of the fractions

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a powerful technology applied for the identification and quantification of proteins from biological samples.

3.14.1 Instrument and settings

LC-MS/MS analysis was performed at the Sandor Life Sciences Pvt Ltd., Banjara Hills, Hyderabad. Label-free LC-MS/MS was performed using nano Acquity Waters UPLC system. The samples were passed through the 75 µM x 150 cm x 1.7 µM BEHC18 column with 150 min separation time. The peptide tolerance adjusted was 20 ppm and the fragment tolerance was 30 ppm with the carbamidomethyl and oxidation modifications. The ProteinLynx Global

SERVER^{TM} (PLGS) was used for the quantification and the proteins were identified by using UNIPROT database.

3.14.2 Sample preparation

For the LC-MS/MS analysis, the biological replicates of $1x10^7$ J774A.1 murine macrophages in DMEM were seeded into 100 mm tissue culture plates and incubated overnight for adherence. The late log phase *L. donovani* promastigotes (DD8 and BHU875) were harvested and resuspended in DMEM, and $1x10^8$ parasites were added to the cell culture. This condition was incubated for about 4-6 h for allowing the infection and washed with DMEM, and fresh media is added to both the conditions (i.e. DD8 infected cells and BHU875 infected cells). After washing, the infected cells were incubated for 24 h. Thereafter, these infected cells were harvested and washed with 1X PBS. The resulting cell pellets were lysed with 1X RIPA buffer (150 mM NaCl, 1% NP-40/Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, and 50 mM Tris pH8.0) on ice for about 30 min. These lysed cells were centrifuged at 6000 rpm for about 15-20 min and the protein lysate in the supernatant is collected for LC-MS/MS analysis. Thus, protein lysates from 4 biological replicates were prepared in a similar manner as described above.

3.15 Cloning, expression, and purification of *L. donovani* FeSODA

3.15.1 PCR amplification of FesodA

The FesodA gene (LdBPK_080300.1) was amplified from genomic DNA using Phusion High-Fidelity Taq by PCR in Veriti 96-well thermal cycler (Applied Biosystems). The forward and reverse primers were designed by inserting the EcoRI and HindIII restriction sites at their 5' end respectively. The primer sequences designed are:

Forward primer 5'-ATAGAATTCATGTTCCGCCG-3' and

Reverse primer 5'-AGCAAGCTTTTACTTCGTGG-3'

The 25 µl PCR reaction mixture included 1 µg DNA, 0.2 mM dNTP, 10 pmol each of both forward and reverse FesodA primers, and 1 U of Taq DNA polymerase. The reaction program was carried out as follows: initial denaturation at 95^{o}C for 5 sec; 35 cycles of final denaturation at 95^{o}C for 30 sec, annealing at 53^{o}C for 45 sec, and extension at 68^{o}C for 1 min; and final extension at 68^{o}C for 10 min. After the reaction, the PCR amplicon was subjected to gel electrophoresis on 1% agarose gel, stained with EtBr, and the band was visualized under the UV transilluminator. After the confirmation, this PCR product was purified using Macherey-Nagel's

PCR clean up/Gel extraction kit as per manufacturer's instructions. Thus purified PCR products were then double digested with EcoRI and HindIII, and ligated with pET28a expression vector.

3.15.2 Double digestion

The FesodA PCR amplicon and the pET28a vector were double digested with EcoRI and HindIII for about 3 h at 37^{o}C. These double digested products were run on 1% agarose gel electrophoresis and purified from the gel.

3.15.3 Ligation

The double digested FesodA insert and pET28a vector were mixed in a ratio of 3:1 molar ratio in the presence of T4 DNA ligase. The ligation reaction mixture included pET28a vector, FesodA insert DNA, 1 U T4 DNA ligase and Milli-Q. The ligation was carried out at 22^{o}C for 16 h.

3.15.4 Preparation of Competent Cells

The competent cells of *E. coli* strains, DH5α and BL21(DE3) were prepared as described further below. The single colonies of either strain mentioned previously were inoculated in 5-10 ml LB broths separately. These inoculated broths were grown at 37^{o}C for overnight with continuous and vigorous shaking. Later, 50 ml secondary cultures were inoculated with the primary culture and grown at 37^{o}C until their OD_{600} reaches to 0.4-0.5. These cultures were then transferred to propylene tubes and let it cool on ice. Then, they were harvested at 2700 x g for 10 min at 4^{o}C. The bacterial pellets were resuspended in ice-cold $MgCl_2$-$CaCl_2$ solution and centrifuged at 2700 x g for 10 min at 4^{o}C. The pellet was finally resuspended in 2 ml ice-cold 0.1 M $CaCl_2$ with 15% glycerol. Thus prepared competent cells were aliquoted into several tubes of 100 µl each and were stored at -80^{o}C.

3.15.5 Determination of transformation efficiency

pET28a plasmid vector (10 ng) and competent cells (100 µl) were mixed and subjected to heat shock at 42^{o}C for 90 sec. To this, 400 µl LB broth was added and different aliquots were plated onto LB agar plates containing kanamycin. The plates were incubated overnight at 37^{o}C. After the emergence of colonies on the plates, the transformation efficiency was calculated as a number of transformants/µg of DNA used.

3.15.6 Transformation of cloned vector

The transformation of cloned vector (i.e. vector ligated with the desired insert) was initiated with the thawing of a vial of the cryopreserved competent cells on ice. As soon as the competent cells

get thawed, they were mixed with 10-20 ng of vector construct DNA and incubated on ice for 30 min. Later, this mixture of competent cells and vector DNA was subjected to heat shock at 42^{o}C for 90 sec. It was immediately placed on ice for about 5 min to which 600-800 µl of LB broth was added. Then it was incubated at 37^{o}C for 30-45 min for allowing the expression of antibiotic resistant genes. These cells were centrifuged at 3000 rpm for 5 min, and the supernatant was discarded. The cell pellet was resuspended in the residual LB medium (50-100 µl) and spread onto the LB agar plates containing kanamycin.

3.15.7 Plasmid isolation and restriction digestion

The selected transformant colonies were inoculated into 5-10 ml LB broth with 50 µg/ml kanamycin and grown overnight at 37^{o}C with continuous shaking at 250 rpm. These overnight cultures were centrifuged at 5000 rpm for 5 min. The obtained cell pellet was used for plasmid isolation. The plasmid isolation was carried out by using Nucleospin plasmid isolation kit according to the manufacturer's instructions. The quality of isolated plasmid DNAs was checked on 1% agarose gel, and their concentrations were estimated using Nanodrop instrument. To confirm the presence of insert in these plasmids, 1 µg of plasmid DNA was mixed with 1 U each of EcoRI and HindIII restriction enzymes along with 1X Tango buffer. This mixture was incubated at 37^{o}C for 3 h to allow the digestion of the plasmid DNAs. Thus digested plasmids were loaded onto the 1% agarose gel and subjected to electrophoresis. The gels were stained with EtBr and observed under UV transilluminator for the presence of insert to confirm the transformants. The confirmed transformants were then cultured in bulk for the higher yield of plasmids by using the Nucleospin plasmid isolation kit.

3.15.8 Gene sequencing

The plasmids with the integrated insert were then sent for sequencing at Xcelris Company to rule out the introduced mutations and to confirm the identity with *L. donovani* FesodA gene.

3.15.9 Recombinant protein expression

The expression of recombinant FeSODA protein was induced as follows. The *E. coli* BL21 (DE3) strain was transformed with the FesodA containing pET28a plasmid construct. Thus transformed *E. coli* BL21 (DE3) cells were plated onto LB agar plate containing kanamycin and incubated overnight at 37^{o}C. A single colony was selected and picked from this plate, inoculated into 5 ml LB broth, and incubated overnight at 37^{o}C with a continuous shaking of 180 rpm. A

secondary culture was inoculated with a small amount of primary culture and grown at similar conditions until the OD_{600} reaches 0.4-0.5. At this point, an aliquot of the culture was separated which serves as an uninduced control. Subsequently, the remaining culture was induced with 1 mM IPTG for different time periods (maximum of 7 h), and an aliquot of culture was collected at every hour. All the samples collected at different time intervals were pelleted down and the cells were lysed in 1X SDS buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 2% SDS, 1% βME, and 0.1% bromophenol blue) and boiled the samples for about 3-5 min. These lysed induced samples of different time intervals subjected to SDS-PAGE (12% gel) (*Laemmli 1970*) after which they were analyzed by Coomassie staining.

3.15.10 Determination of protein solubility

The log phase E. coli culture was induced with 1 mM IPTG at 37^{o}C for about 3 h. These induced cells were collected at 3500 x g for 10 min at 4^{o}C. The pellet was lysed in 5 ml lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 5 mM βME, 1% Triton X-100, and 10% glycerol). 1 mM PMSF and 5 μg/ml DNase were added to the lysate prior to the sonication. The lysate was sonicated at 40% amplitude with 30 sec on and off cycle for 3-5 pulses. This lysate was centrifuged at 12,000 rpm for 30 min at 4^{o}C. The supernatant (soluble fraction) was separated from the pellet (insoluble fraction). The insoluble fraction was resuspended in 1 ml lysis buffer. Both the soluble and insoluble fractions were analyzed by 12% SDS-PAGE.

3.15.11 Protein purification

3.15.11.1 Lysate preparation

A colony of *E. coli* BL21(DE3) was picked from LB agar plate containing 50 μg/ml kanamaycin, inoculated the 100 ml primary culture and was grown overnight at 37^{o}C with a continuous shaking of 180 rpm. Thus overnight grown primary culture was used to inoculate the 1 L secondary culture with 50 μg/ml kanamycin. The secondary culture was grown at 37^{o}C till its OD_{600} reaches 0.4-0.5. Once this OD_{600} was attained, the culture was induced with 1 mM IPTG for 3 h at 37^{o}C. Thus induced cells were harvested at 4000 x g for 5-10 min at 4^{o}C. The pellet was resuspended in lysis buffer and sonicated as described before. The lysate was collected, centrifuged at 12,000 rpm for 30 min at 4^{o}C, and the supernatant was used for further purification.

3.15.11.2 Column preparation and protein elution

Prior to the purification, the Ni^{+2} beads were processed as described further. At first, the Ni^{+2} bead slurry was mixed gently. A small amount of resin was aspirated, washed once with ddH_2O, and twice with binding buffer (50 mM Tris pH 8.0, 0.5 M NaCl, 8M Urea, and 20 mM imidazole) at 5000 rpm for 2 min to remove ethanol. To these beads, the cell lysate was added, and this mixture was end-over rotated for about 1-2 h at RT to allow the binding of Ni^{+2} beads to His-tagged recombinant FeSODA. Followed by the incubation, the mixture was loaded onto the PD-10 desalting column (GE Healthcare Life Sciences). Then the flow through was passed out by adjusting the flow rate to 10 column volumes per hour. Later, the column was washed for 4 times with the washing buffer (50 mM Tris pH 8.0, 0.5 M NaCl, 8 M Urea, and 20 mM imidazole). Finally, the protein was eluted in by passing the elution buffer (20 mM Tris pH 7.5, 100 mM NaCl, 8 M Urea, and 50 mM imidazole).

3.15.11.3 Dialysis

The eluted recombinant protein was subjected to dialysis using SnakeSkin® dialysis tubing (3.5K MWCO). Briefly, the tubing length was determined as described below.

First the tube length was determined from the formula:

Tube length (mm) = Vol (in µl) x 3.2 ÷ flat $width^2$ (in mm^2).

To this, 10% of the tube length obtained from this formula was added. In addition to this, 40 mm was added for the closures.

The length of the dialysis tubing cut according to the above measurements was immersed in the dialysis buffer (25 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol). Then it was closed tightly at one end with a dialysis clamp, and the protein sample was loaded into the tubing from the other open end to check for any leakages. Once no leakage was ensured, the tubing was closed tightly from the other end too. First, 6 M Urea was added to the dialysis buffer and the dialysis tubing with the protein sample was immersed into this buffer. Further, the sample was allowed to dialyze against the buffer for about 2-4 h at RT. Subsequently, the urea concentration was decreased to 4 M, 2 M, and 0 M for every 2-4 h. Finally, the protein sample was collected from the dialysis tubing and stored at -80^{o}C for further use.

3.16 Protein estimation

3.16.1 Bradford method

The protein concentration was determined from the Bradford assay (*Bradford 1976*). Firstly, a standard curve was plotted using BSA as a standard. From the 0.2 mg/ml working concentration of BSA solution in water, different concentrations (2-20 µg) of BSA were taken in triplicates. To these, ddH_2O was added to make up the volume to 100 µl. This solution was then mixed with 1.5 ml Bradford reagent and incubated at RT for 5 min. The Bradford reagent was taken as a blank solution. After the incubation, the color absorbance was measured at 595 nm using a spectrophotometer. Then the standard curve was plotted from which the unknown concentrations of protein samples were deduced (*Deutscher 1990*) .

3.16.2 Amido Black method

The concentrations of 2D protein samples were determined using Amido Black staining method (*Henkel and Bieger 1994*). Briefly, the BSA (1 mg/ml) was used as a standard. The standard was spotted accordingly in duplicates onto an NC membrane. Then 2 µl of test samples were spotted in triplicates. The spots were allowed to dry. Later the spots were incubated with Amido Black solution (0.1% amido black, 10% acetic acid, 45% methanol) for about 30 min. After the staining, the membrane was destained with destaining solution (90% methanol, 2% acetic acid, 8% Milli-Q) for 30 min until the spots are visible. Once the spots get visible, they were excised and dropped into the elution buffer (50% ethanol, 0.2 N NaOH, 50 mM EDTA) in tubes. Then they were allowed to elute into this elution buffer by intermittent vortexing. The blank solution used was the elution buffer. Finally, the absorbance was read at 630 nm and the unknown concentrations of the test samples were deduced from the standards.

3.17 Antibody raising in mice

The antibody against FeSODA was raised in mice as described here. The recombinant FeSODA protein was dialyzed, and the concentration was estimated using Bradford assay. About 40 µg of recombinant FeSODA was mixed with an equal volume of Freund's complete adjuvant. Then this mixture was vigorously mixed for several times with a sterile syringe needle to prepare an emulsifier. This emulsifier was then subcutaneously (SC) injected into BALB/c mouse. Later, on the 14^{th} day, the mouse was SC injected with the first booster dose prepared by the emulsification of 40 µg FeSODA and Freund's incomplete adjuvant. On the 21^{st} day, the second

booster was prepared in a similar manner and SC injected in mice. Finally, on the 30th day, the whole blood was drawn from the mouse retro-orbitally. The blood was allowed to clot by leaving it undisturbed for about 10-15 min at RT. The serum was separated by centrifuging at 2000 x g for 10 min at 4^{o}C. Finally, serum was collected in a fresh tube and stored at -80^{o}C until further use.

3.18 Transfection of *Leishmania*

The transfection of *Leishmania* parasites was done as described previously *(Beverley and Clayton 1992*).

3.18.1 Electroporation

Briefly, the parasites (DD8 and BHU875) were grown in sufficient M199 medium till they reached late log phase cells. Once the parasites reached the late log phase, the plasmid pEGFP-C1 and pEGFP-C1 with FeSODA insert along with 2 mm electroporation cuvettes were placed on ice. The parasites were harvested at 1000 x g for 5 min. The supernatant was discarded and the parasite pellets were resuspended in 5-10 ml filter-sterilized electroporation buffer or EPB (21 mM HEPES pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM Glucose) until their complete dispersion. The parasites were washed again with EPB. Subsequently, the parasites were resuspended at a density of 10^8 per ml of EPB and immediately placed on ice. From this parasite suspension, $4x10^7$ parasites (i.e. 0.4 ml) were aliquoted into each cuvette and left on ice for a few minutes. Meanwhile, Gene Pulser was turned on, and the voltage was set to 0.45 kV voltage and 500 μF capacitance with 3.5-4.5 ms constant pulse time. Hereafter, the 10-50 μg plasmid DNAs were added to the parasite suspension in electroporation cuvettes and mixed well with sterile Pasteur pipette. The cuvettes were placed in the electroporator chamber, subjected to the pulse, and then placed on the ice immediately. After 10 min incubation on ice, the electroporated parasites were transferred to 5-10 ml of M199 medium devoid of G418 and incubated for 16-24 h.

3.18.2 Preparation of M199 selective plates

M199 selective plates were prepared as described further. Briefly, 2X M199 media powder was dissolved in sterile Milli-Q water supplemented with 2.4 μg/ml biopterin. Simultaneously, 2% agar was added to sterile Milli-Q water, autoclaved to melt the agar, and then cooled to 55^{o}C. Meanwhile, the 2X M199 media was incubated at 37^{o}C. When the 2% agar temperature was

brought down, the 100 μg/ml G418 was added to the warm 2X M199 followed by the addition of an equal volume of agar. This mixture of 2X M199 and 2% agar was mixed gently, and pipetted onto the 100 mm petri plates by avoiding the air bubbles. After the solidification of the medium, the uncovered plates were dried by a brief exposure (~10-15 min) in a sterile hood (Alternatively, the covered plates were dried on the bench overnight). The pH of the solidified media was equilibrated by incubating the poured plates in a 5% CO_2 incubator for 2-4 h just prior to their use.

3.18.3 Harvesting and plating Leishmania for stable transfections

The overnight incubated electroporated parasites were transferred into a 15 ml tube and collected at 1000 x g for 5 min. The supernatant was carefully discarded, and the loose pellet was resuspended in the residual M199 in the tube by flicking with a finger. This suspension was then transferred and gently spread onto the M199 selective plates. The plates were then wrapped in a parafilm and placed at 25^{o}C with agar side facing upwards. After the emergence of colonies, a colony was picked using sterile pipette tips, and inoculated into 1 ml M199 medium containing 50 μg/ml G418 drug in 24-well plates. After the dense growth of the parasites, they were transferred into culture flasks.

3.18.4 Selection of transfectants in liquid culture

The *Leishmania* transfectants were selected according to the method previously described (*Taheri, Gholami et al. 2014*). After the 16-24 h incubation in G418-free M199 medium, the transfectants were subjected to 20 μg/ml G418 pressure for about 5-6 days. Then, the selected transfectants were exposed to 50 μg/ml G418 pressure and used for further studies.

3.19 Immunofluorescence assay

IFA was carried out as described previously *(Selvapandiyan, Duncan et al. 2001*). The *Leishmania* parasites were fixed in suspension of 4% (w/v) paraformaldehyde in PBS at RT for 20 min. Thus fixed parasites were then washed thrice in PBS, allowed to attach to the cover slips on glass slides, and air dried. These dried cover slips were once flooded with ice-cold methanol (-20^{o}C) for 5 min. Then they were blocked with 1% (w/v) BSA in PBS for 30 min. This was subsequently followed by the 1 h incubation with primary antibody i.e. anti-FeSODA (1:200) diluted in 1% BSA in PBS. After the incubation, they were washed thrice with PBS, and incubated with the Goat anti-mouse IgG secondary antibody conjugated with the fluorochrome

Alexa Fluor® 594 (1:200). The coverslips were again washed thrice with PBS. Thus processed coverslips were mounted in Vectashield antifade mounting medium with DAPI onto the glass slides. These were further observed under the confocal microscope for the fluorescence.

3.20 Flow cytometric analysis of viability using PI

To check the effect of FesodA knockdown on miltefosine resistance, the viability of BHU875 and BHU875 KD (i.e. FeSODA depleted BHU875) parasites was analyzed by staining with PI. Briefly, $2x10^6$ log phase promastigotes were treated with varied concentrations (0 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, and 60 μM) miltefosine for about 48 h. After incubation, all the parasites were collected at 2000 rpm for 5 min. The pellet was washed with PBS and resuspended in PBS. These resuspended parasites were stained with 5 μg/ml PI for 5 min, and analyzed using BD LSRFortessa^{TM} using FACSDiva software.

3.21 Preparation of soluble *Leishmania* antigen (SLA) from promastigotes

SLA was prepared from the promastigotes of DD8 and BHU875 *L. donovani* strains as described previously (*Saha, Nanda-Roy et al. 1991*). Briefly, the late-log phase promastigotes were harvested at 4000 rpm for 20 min at 4^{o}C. The parasite pellets were washed twice with 1X ice-cold PBS. The resultant pellet was dissolved in 180 μl Milli-Q water, and subjected to five cycles of freezing and thawing at -80^{o}C. To this, 1X protease inhibitor cocktail was added and vortexed for few seconds. Followed by this, it was immediately placed on ice, and sonicated at 20% amplitude with 5 cycles of 45 sec on and 1 min off. Immediately after the sonication, the lysate was centrifuged at 9980 x g for 15 min at 4^{o}C to remove the debris. The resultant SLA in the supernatant was collected and stored at -80^{o}C till further use.

3.22 SDS–PAGE and Immunoblot analysis

The protein samples were subjected to electrophoresis on 12% SDS-PAGE until the sample dye reaches to the bottom of the gel. These separated proteins were transferred onto a nitrocellulose membrane for about 14-16 h at 35 V, 4^{o}C. The NC membrane was then stained with Ponceau S to verify the transfer of proteins. After the confirmation of protein transfer, the Ponceau S stain was removed by washing the membrane with ddH_2O repeatedly. Then the membrane was blocked in a blocking buffer [5% (w/v) skimmed milk in TBST (i.e. 1X TBS with 0.05% Tween-20)] for about 2-3 h at RT. Later, the blot was incubated with primary anti-mouse FeSODA antibody (1:100) diluted in blocking buffer overnight at 4^{o}C. Further, the blot was washed thrice

with 1X TBST for about 10-15 min each. Subsequently, the blot was incubated for 1 h with Rabbit anti-mouse IgG secondary antibody conjugated to ALP (1:10,000) diluted in blocking buffer. Followed by this incubation, the blot was again washed thrice with 1X TBST for 10-15 min each. After the washes, the blot was treated with ECL reagent, and the protein bands were visualized by exposing it to UV radiation.

Chapter 4:

RESULTS

4.1 MTT assay determines the higher IC_{50} of miltefosine for BHU875 resistant parasites

The differential cytotoxic effect of miltefosine against DD8 and BHU875 *L. donovani* strains was assessed by MTT colorimetric assay. The mitochondrial succinate dehydrogenase of living cells reduces the yellow tetrazole MTT to insoluble, purple formazan crystals (*Mosmann 1983*). The solubilization solution DMSO dissolves the insoluble purple formazan crystals into a colored solution. The absorbance of this colored solution was quantified by measuring at 540 nm. When both the parasite strains were treated with different concentrations of miltefosine for 72 h, miltefosine was found to have varied cytotoxicity towards both the strains. It was found to be highly cytotoxic for DD8 parasites with IC_{50} ranging between 15±5 µM, and the miltefosine effect on the clinical resistant strain showed upto 80 µM concentration. This observation indicated that BHU875 indeed displayed the resistant phenotype *in vitro*.

A

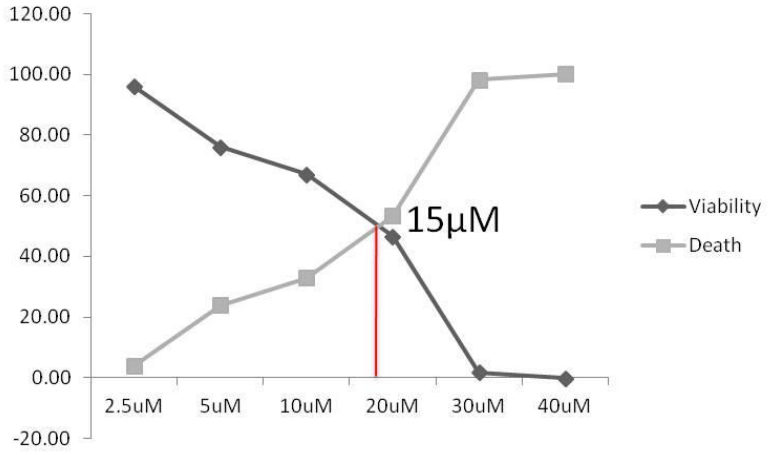


Fig 4.1A Determination of Miltefosine IC_{50} on DD8 parasites by MTT assay.

B

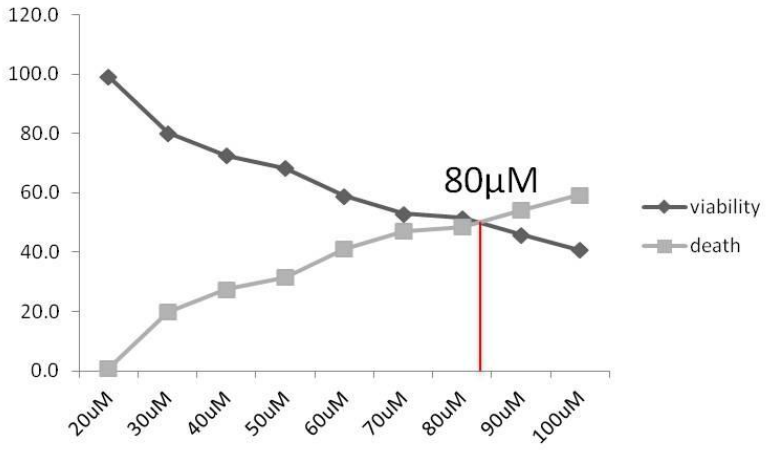
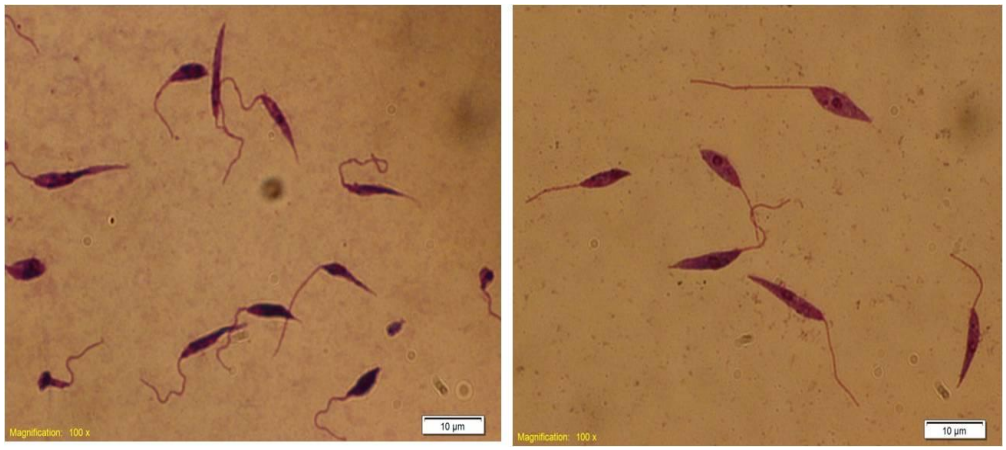


Fig 4.1B Determination of Miltefosine IC_{50} on BHU875 parasites by MTT assay.

4.2 Miltefosine resistance has not altered the parasite morphology

To investigate the morphological differences in the DD8 and BHU875 parasites, Giemsa staining was performed. The microscopic observation of these stained parasites pointed out that BHU875 exhibit regular morphology i.e. slender and flagellated similar to that of DD8 sensitive parasites.

A



B

Fig 4.2 Morphological comparison of *Leishmania* parasites by Giemsa staining. (A) DD8 and (B) BHU875.

4.3 Cell cycle was not stalled in BHU875 parasites with miltefosine treatment

Cell cycle analysis gives an insight into the status of proliferation. Any alteration in the progress of cell cycle indicates the damage to the cells. Usually, the living cells exclude PI. Hence, cell permeabilization with 70% ice-cold ethanol fixation helps the accumulation of PI inside the cells. In this direction, both the DD8 and BHU875 parasites were treated with 40 µM miltefosine for different time points. The following histogram depicts the DNA content of the parasites. The 1st quadrant denotes sub G0/G1, 2nd quadrant denotes G1 phase, 3rd quadrant denotes S phase, and 4th quadrant denotes G2/M phase. The miltefosine treated DD8 parasites showed defects in the growth pattern; most of the cells were found to be amassed in the sub G0/G1 phase with the increase in time. In contrast, BHU875 parasites exhibited undisturbed proliferation with miltefosine treatment. This result validates that the miltefosine was unable to stall the cell cycle of resistant parasites unlike that of sensitive parasites.

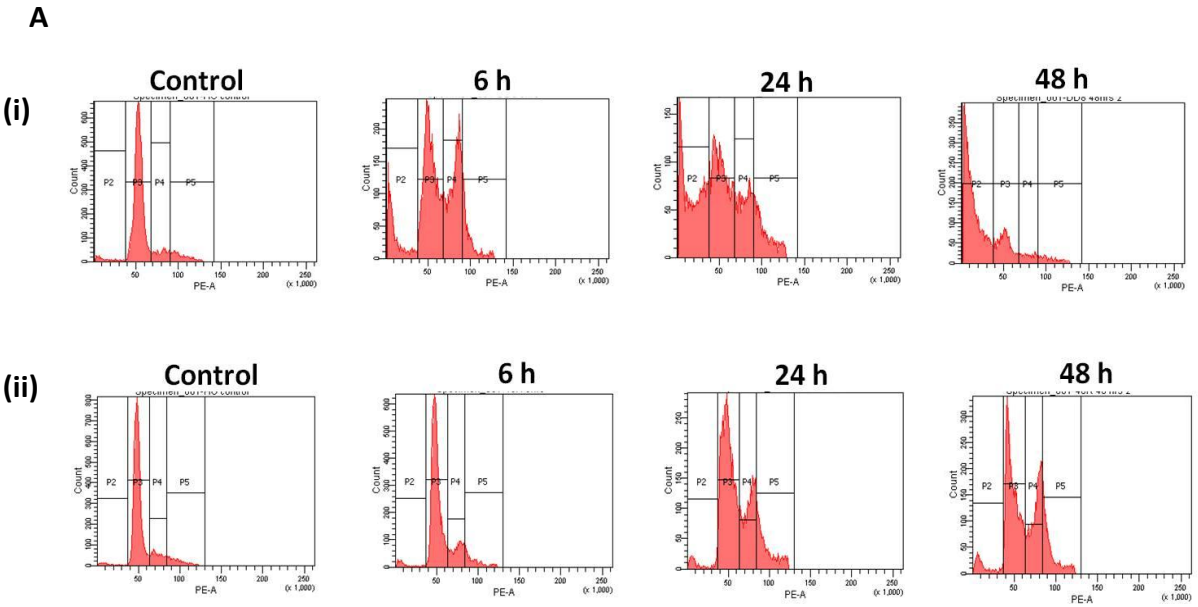


Fig 4.3A Histogram depiction of *Leishmania* parasites' cell cycle analysis by flow cytometry using propidium idodide. (i) DD8 parasites treated with 40 µM Miltefosine. (ii) BHU875 parasites treated with 40 µM Miltefosine.

B

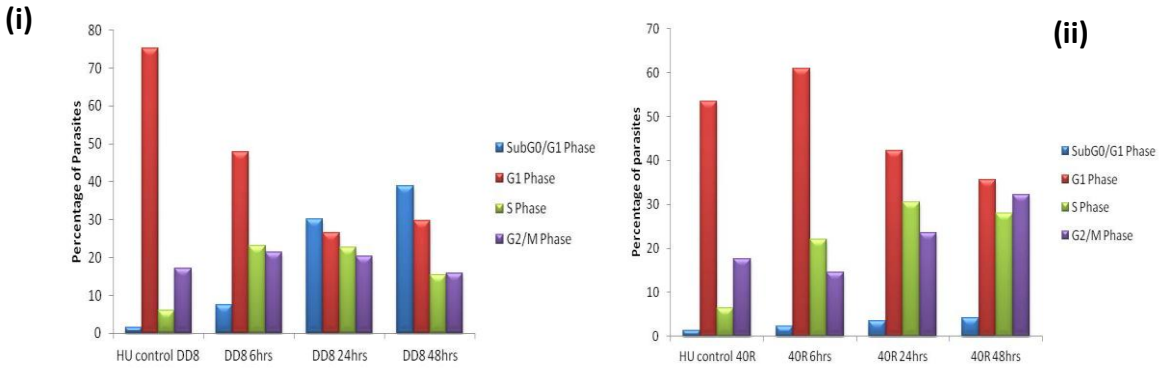


Fig 4.3B Bar graph depiction of *Leishmania* parasites' cell cycle analysis by using propidium iodide. (i) Miltefosine treated DD8 parasites at 48 h (ii) Miltefosine treated BHU875 at 48 h.

4.4 Resistant parasites render miltefosine unable to induce ROS

Miltefosine is known to induce apoptotic-like features in *L. donovani* promastigotes *(Paris, Loiseau et al. 2004*). Towards this direction, the efficacy of miltefosine in the production of ROS was studied in the case of both DD8 and BHU875 parasites. The ROS production was estimated using a cell permeant dye, H_2DCFDA. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The red fluorescence intensity is directly proportional to the ROS levels. The flow cytometry analysis of untreated and 40 μM miltefosine treated parasites revealed an elevated ROS levels in treated DD8 parasites. But the BHU875 parasites showed the similar amount of ROS levels with and without treatment. This data clearly signifies that the BHU875 is showing resistance against miltefosine.

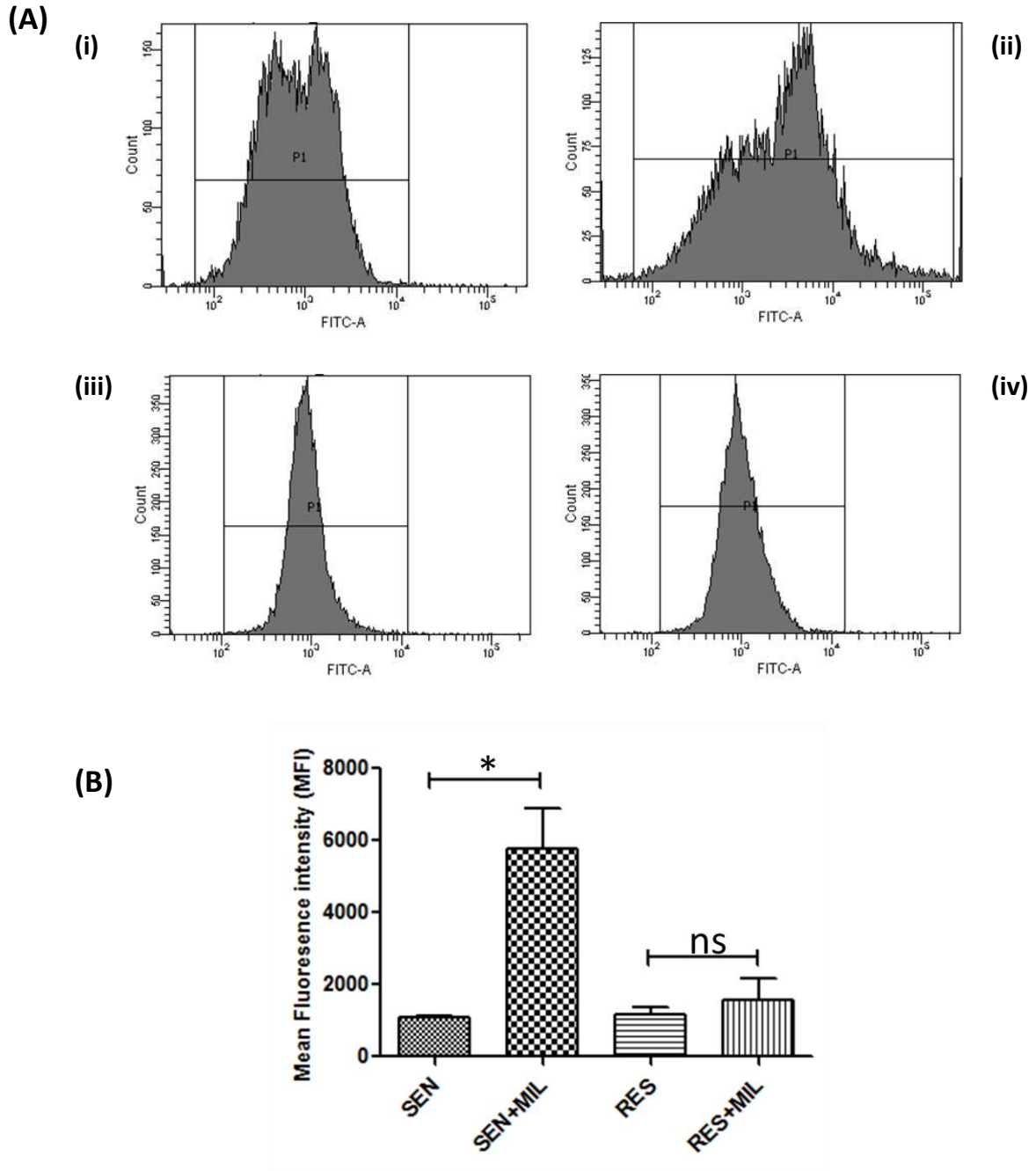


Fig 4.4 Determination of reactive oxygen species (ROS) in *Leishmania* parasites by flow cytometry. (A) Histogram depiction of (i) Untreated DD8 parasites, (ii) Miltefosine treated DD8 parasites, (iii) Untreated BHU875 parasites, (iv) Miltefosine treated BHU875 parasites. (B) Bar graph depiction of ROS in terms of mean fluorescence intensity (MFI).

4.5 Miltefosine treatment fails to reduce the intracellular parasite burden

The effect of miltefosine on intracellular resistant parasite was analyzed in terms of parasite burden. The parasite burden was observed by counting the amastigotes number through Giemsa staining. The J774A.1 murine macrophages were infected with sensitive and resistant parasites for 6 h, and treated with 1 μM miltefosine. The number of amastigotes was counted per 100 macrophages of both treated and untreated macrophages. The results demonstrated that the treatment with miltefosine reduced the sensitive parasite number, but the resistant parasite number remained constant as that of untreated. This data indicates the miltefosine treatment had no effect on the intracellular parasite burden.

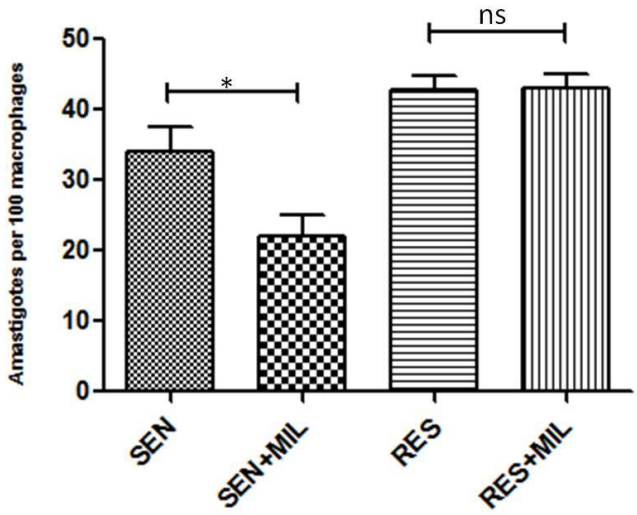


Fig 4.5 Amastigote counting in *Leishmania*-infected J774A.1 macrophages by Giemsa staining.

4.6 Miltefosine resistant parasites down regulate the IFN-γ gene expression levels without altering the IL-10 levels

IFN-γ and IL-10 are the major cytokines that play a predominant role in determining the outcome of visceral leishmaniasis. IFN-γ reduces the disease outcome whereas IL-10 promotes the intracellular parasite survival. The quantitative real-time PCR analysis of IFN-γ and IL-10 was performed for the macrophages infected with sensitive and resistant parasites. The results

displayed the down regulation of IFN-γ in the macrophages infected with resistant parasites. But, in contrast, the IL-10 levels were similar in the case of both the macrophages infected with sensitive as well as resistant parasites. The down regulation of IFN-γ, without any modulation in IL-10 levels, explains that the miltefosine resistant parasites significantly suppress the Th1 immune response compared to that of sensitive parasites.

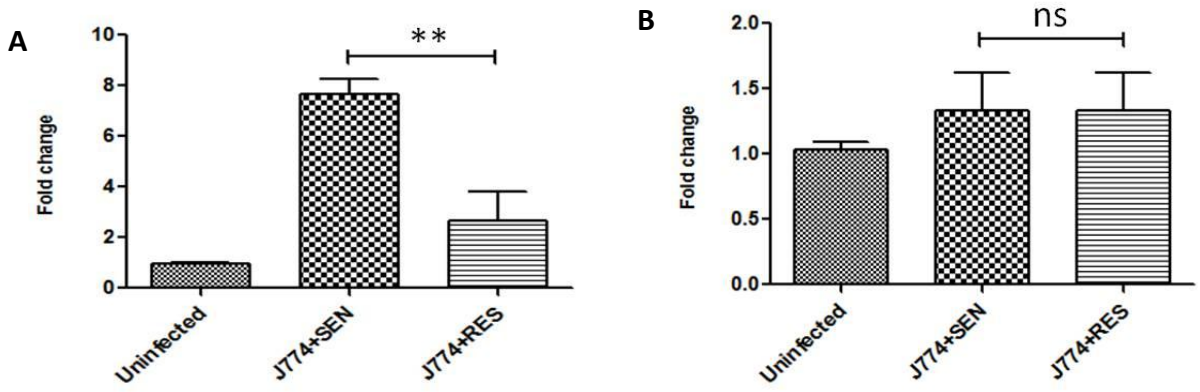


Fig 4.6 Real-time PCR of IFN- γ and IL-10 cytokines in *Leishmania*-infected J774A.1 macrophages. (A) IFN- γ in the macrophages (B) IL-10 levels in the macrophages.

4.7 Miltefosine resistance significantly decreases the IFN-γ/IL-10 ratio

During the visceral leishmaniasis, the IFN-γ/IL-10 ratio determines the fate of intracellular parasite survival. The infection of J774A.1 murine macrophages with resistant parasites revealed a significant decrease in IFN-γ/IL-10 ratio compared to those infected with sensitive parasites. The decrease in IFN-γ/IL-10 ratio symbolizes that there is a pronounced shift of immune response towards Th2 type promoting the intracellular parasite survival.

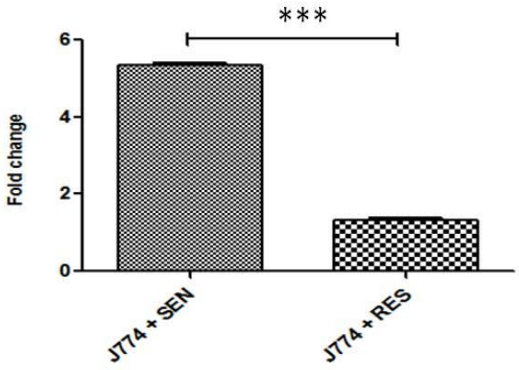


Fig 4.7 Determination of IFN-γ/IL-10 ratio in *Leishmania*-infected J774A.1 macrophages.

4.8 Intracellular resistant parasites evade the NO production in the murine macrophages

The NO production usually mediates the intracellular parasite death. The J774A.1 macrophages were infected with sensitive and resistant parasites for 72 h, and treated with 1 μM miltefosine. The absorbance values obtained from Griess assay depicted that the miltefosine produced a significant amount of NO in case of macrophages infected with sensitive parasites. In distinction with this, the resistant parasites evade the NO production in murine macrophages. This further clarifies that the miltefosine resistant parasites are highly competent in evading the nitric oxide production by miltefosine.

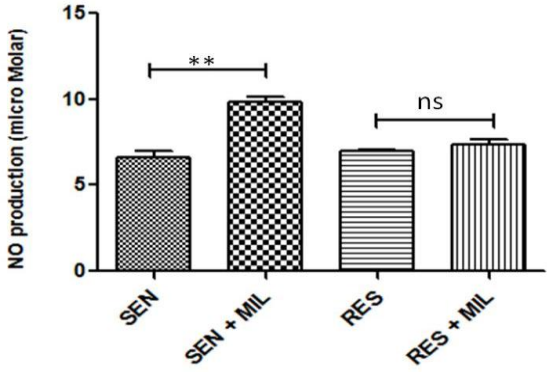


Fig 4.8 Determination of nitric oxide (NO) production in *Leishmania*-infected J774A.1 macrophages.

4.9 Two-dimensional electrophoresis revealed the differentially regulated proteins during miltefosine

The separation of proteins by 2D gel electrophoresis relies on the two factors namely, pI and molecular weight. This helps in the separation of a wide range of proteins from a biological sample. Using the comparative 2D gel electrophoresis approach, the proteomes of DD8 and BHU875 were successfully separated on 12.5% SDS-PAGE preceded by IEF on IPG strip of pH 3-10 range (Fig…). About 12 proteins were found to be differentially regulated from densitometric analysis using ImageJ software. Among these differentially regulated proteins, 4 proteins were up regulated, and 8 were down regulated. The proteins with <1.5 fold change were considered as downregulated and those with ≥1.5 fold change as upregulated.

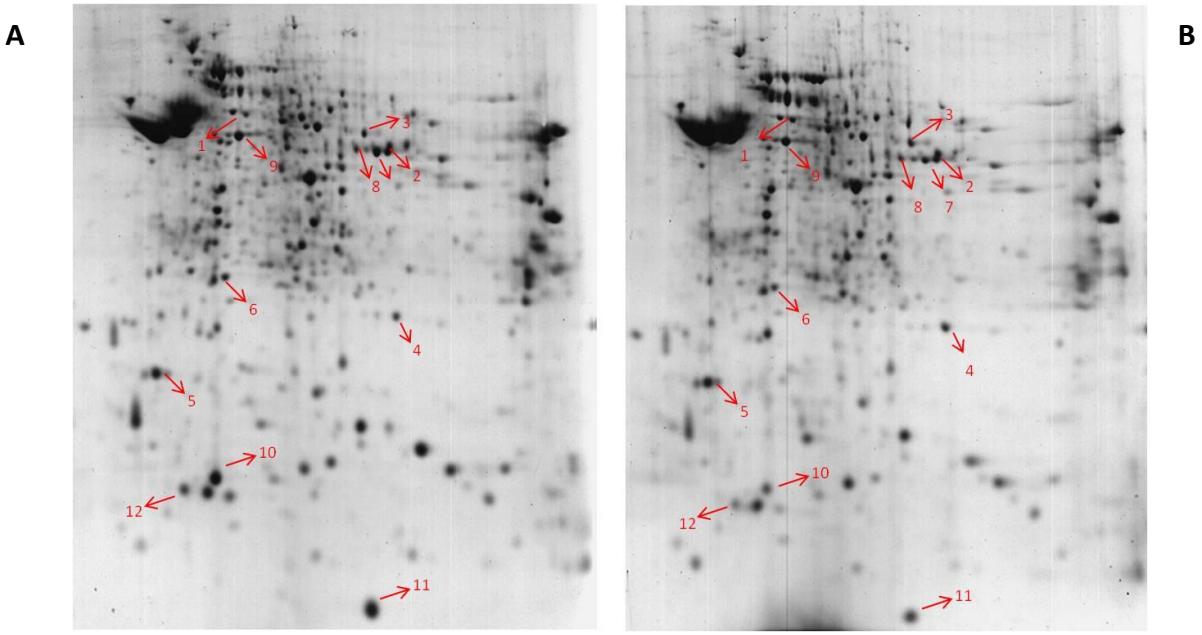


Fig 4.9 Two-dimensional electrophoresis of total lysates of (A) sensitive and (B) resistant parasites. The arrows indicate the differentially regulated proteins in the sensitive and resistant parasites.

4.10 MALDI TOF-TOF and Mascot analysis unveiled the differentially regulated proteins

The differentially regulated proteins were identified by MALDI-MS/MS analysis using Mascot search engine. The molecules in the MALDI matrix are desorbed and ionized by charge transfer by the absorption of energy of a short laser pulse. They are separated based on their mass, and a mass spectrum is created illustrated by the mass and the intensity of the ions. The resulting spectral signatures are used for searching the identification of the proteins from the appropriate database. In our study, 12 differentially regulated proteins were identified as listed in the table from Swiss-Prot database using Mascot search engine with the help of MS/MS spectral signatures.

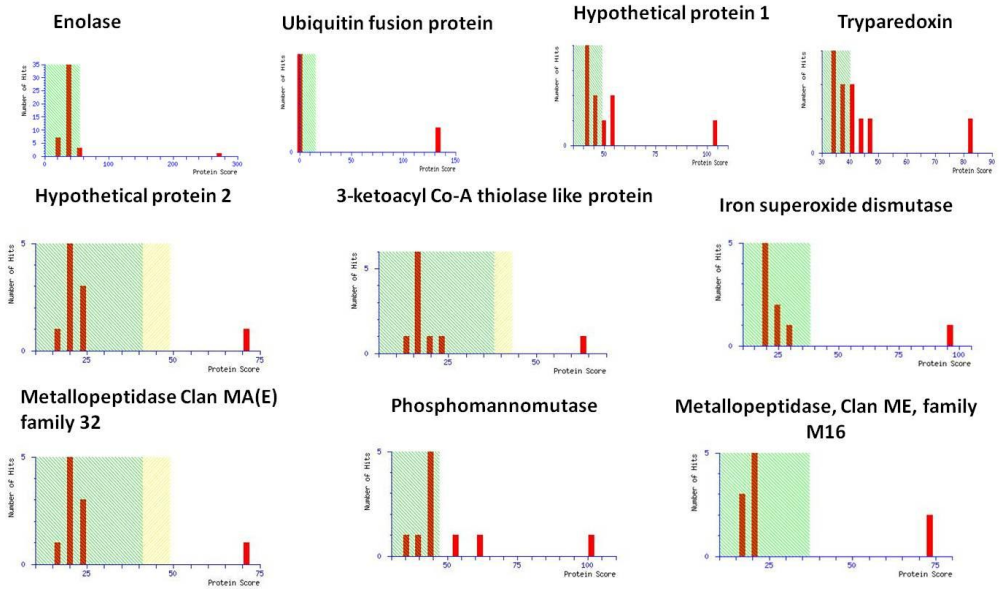


Fig 4.10 MASCOT analysis of the differentially regulated proteins

Spot No.	Protein identified	Species	Accession No.	Sub-cellular localization
1	Metallopeptidase M32	*L. major*	LMJF_13_0090	Cytosolic
2	Hypothetical protein	*L. infantum*	LinJ.27.2220	Unknown
3	Metallopeptidase M16	*L. infantum*	LinJ.35.1390	Mitochondria
4	Iron superoxide dismutase	*L. infantum*	LINJ_08_0300	Mitochondria
5	Hypothetical protein	*L. infantum*	LDBPK_130270	Unknown
6	Phosphomanno mutase	*L. infantum*	LinJ.36.2070	Cytosolic
7	3-ketoacyl co-A thiolase like protein	*L. mexicana*	LMXM_23_0690	Mitochondria
8	3-ketoacyl co-A thiolase like protein	*L. infantum*	LINJ_23_0860	Mitochondria
9	Enolase	*L. donovani*	LdBPK_141240	Cytosolic
10	Tryparedoxin	*L. infantum*	LiINJ_29_1250	Cytosolic
11	Ubiquitin fusion protein	*L. donovani*	LdBPK_311930	Cytosolic
12	Hypothetical protein	*L. infantum*	LINJ_05_0450	Unknown

Table 4.1 List of the differentially regulated *L. donovani* proteins in 2D-gel electrophoresis. Dark gray color – Upregulated proteins; Light gray color – Downregulated proteins.

4.11 Real-time PCR of parasite genes

The quantitative real-time PCR of parasite genes was performed for the differentially regulated genes at protein level from 2D gel electrophoresis. The cDNAs were successfully synthesized and subjected to real-time PCR. The calculated fold changes demonstrated that 4 genes namely, enolase, tryparedoxin, ubiquitin 16S, and phosphomannomutase were significantly down regulated at the gene level. But the other genes such as FeSODA, 3-ketoacyl coA thiolase like protein, hypothetical protein, metallopeptidases M16, and M32 were non-significantly regulated.

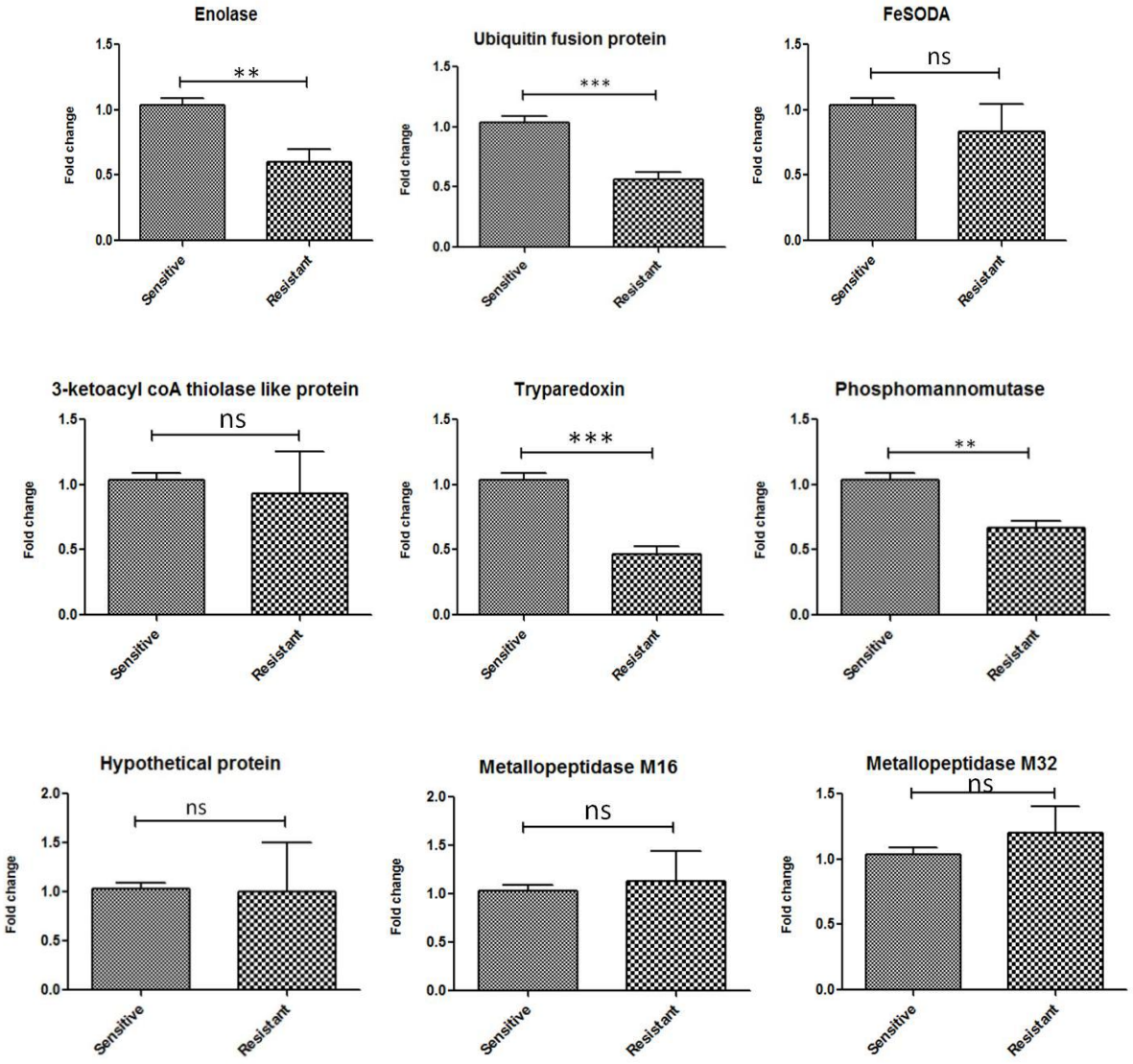


Fig 4.11 Real-time PCR of the differentially regulated genes in the 2D-gel electrophoresis

4.12 FeSODA is conserved amongst the *Leishmania* spp.

The FeSODA protein sequence was subjected to multiple sequence alignment. The results obtained suggest that FeSODA showed 99% homology among the *Leishmania* species.

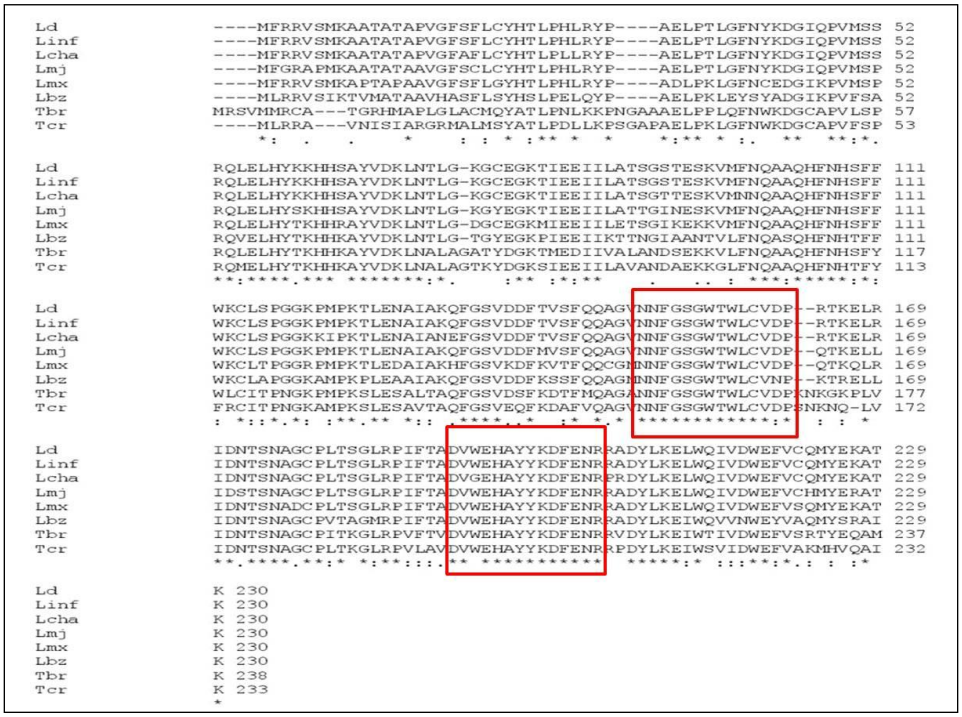


Fig 4.12 Multiple sequence alignment of the FeSODA protein sequence from different *Leishmania* spp. and *Trypanosoma* spp.

4.13 FesodA was cloned into pET28a, and FeSODA was induced in *E. coli* BL21(DE3)

FesodA gene (693bp) was successfully amplified and cloned into pET28a expression vector. The cloning was confirmed by the double digestion of pET28a with EcoRI and HindIII as shown in the below fig. After the successful insertion of FesodA gene into pET28a, the vector construct (pET28a-FesodA) was transformed into *E.coli* BL21(DE3). The FeSODA was successfully induced at all the time points from 1 h induction to 6 h induction. As the expression was found to be high at 3 h time point, the 3 h induction was considered for the purification experiments.

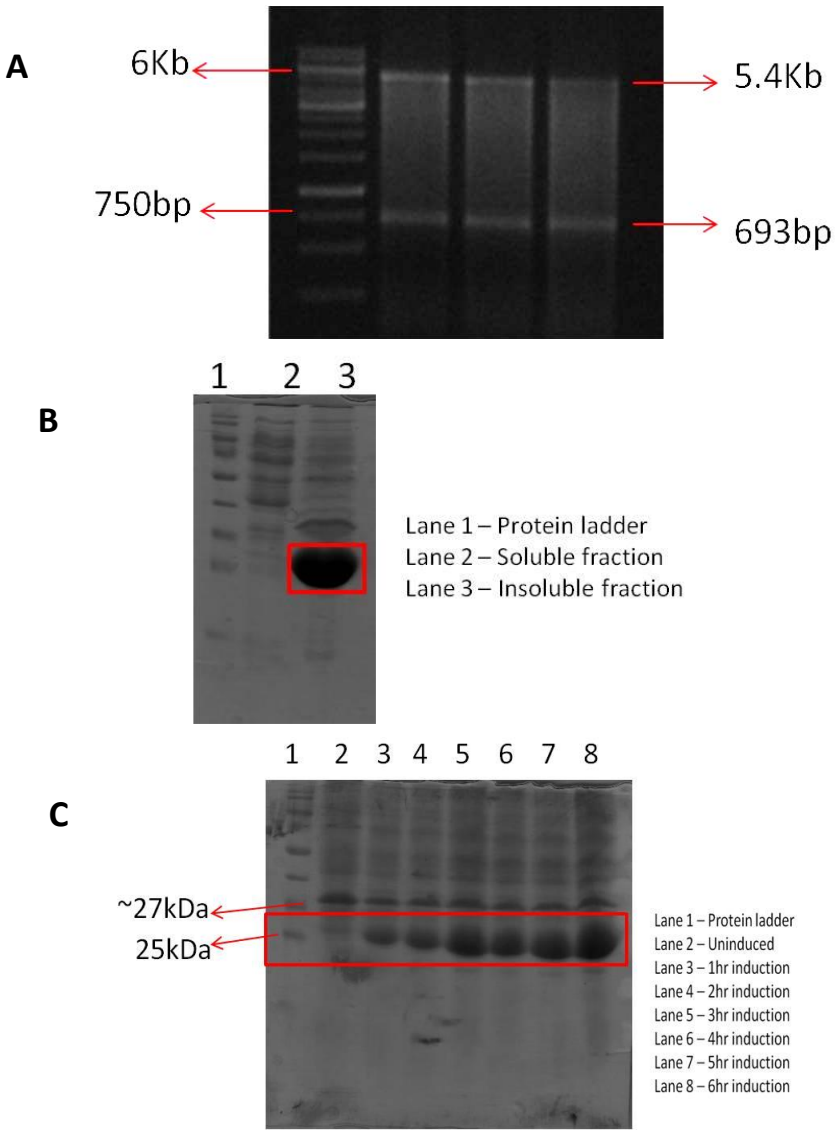


Fig 4.13 Cloning and purification of FeSODA. (A) Double digestion of plasmids cloned with FesodA gene (antisense orientation. (B) Fractionation of *E. coli* BL21 (DE3) lysate – Lane3 (insoluble fraction) shows the presence of FeSODA. (C) Induction of FeSODA with IPTG in *E. coli* BL21 (DE3) for different time points.

4.14 Polyclonal anti-FeSODA Antibody raising and protein levels determination

The polyclonal anti-FeSODA antibody was successfully collected from BALB/c mice. The detection ability of anti-FeSODA against the FeSODA protein was tested. The whole protein lysate was transferred onto an NC membrane, and the FeSODA was successfully detected at 1:100 dilution of anti-FeSODA.

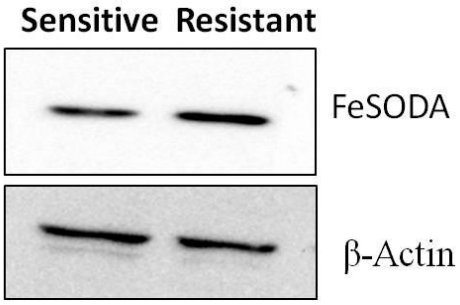


Fig 4.14 Western blot analysis of FeSODA levels in *Leishmania* lysates.

4.15 FesodA was successfully cloned into pEGFP-C1 vector

The FesodA was successfully cloned into pEGFP-C1 using EcoRI and HindIII in an antisense orientation. The insertion was confirmed by the double digestion of pEGFP-FesodA construct.

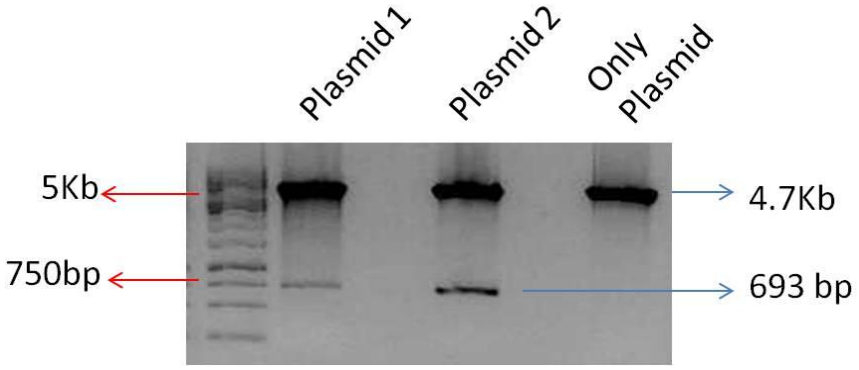


Fig 4.15 Double digestion of FesodA-pEGFP construct. The lane1 and 2 indicate the successful insertion of FesodA (693 bp) and lane3 has no insert.

4.16 *L. donovani* transfectants were selected on liquid M199-agar media

The *L. donovani* transfectants were plated onto liquid M199-agar selective plates, and the colonies appeared after 7-10 days. The white colonies on the plate indicate the *L. donovani* single colonies.

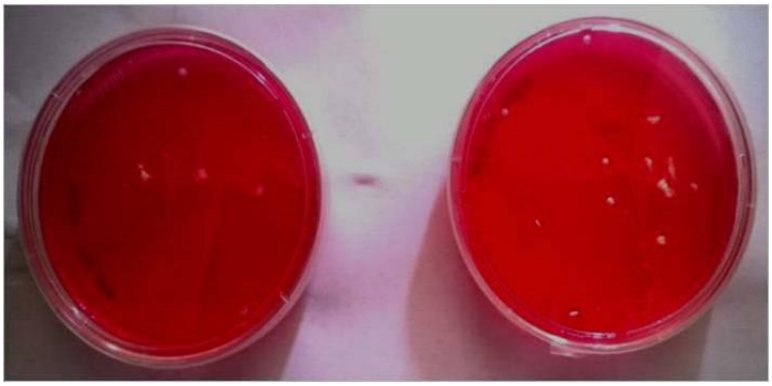


Fig 4.16 Selection of *L. donovani* transfectants on liquid M199-agar media.

4.17 Immunoblotting asserts the successful FeSODA depletion

The FeSODA inhibition was confirmed by the immunoblotting of whole DD8 and BHU875 parasite lysates. The first blot in the following figure indicates the immunoblot of DD8 WT (wild-type) and KD (knockdown) parasites. In this, the detection of FeSODA by anti-FeSODA antibody in the WT indicates the presence of FeSODA protein in the control transfectants (i.e. DD8 parasites transfected with pEGFP-C1). The absence of a band in the 2nd lane or the non-detection of protein band denotes that the FeSODA was successfully depleted in the DD8 FesodA KD parasites. In parallel, the immunoblot of BHU875 resistant parasites was also depicted in the second figure. This figure reveals the successful depletion of FeSODA in BHU875 KD parasites as no protein band was detected in the 2nd lane in addition to a successful detection of the protein in the 1st lane.

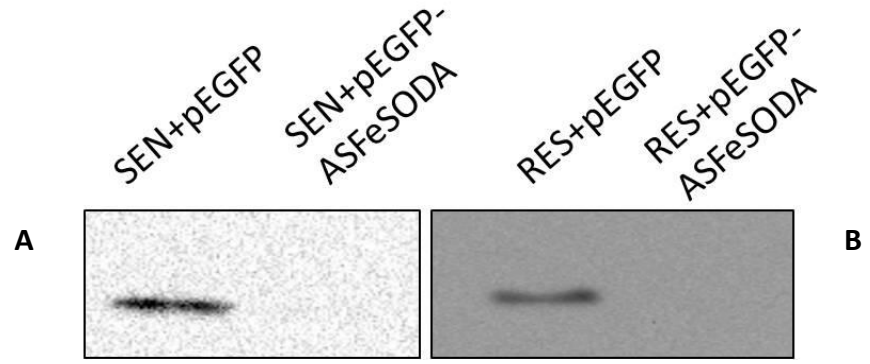


Fig 4.17 Immunoblot analysis of *L. donovani* transfectants with anti-FeSODA antibody. (A) Immunoblot of sensitive parasite transfectants' total lysates. Lane 1 - sensitive parasites transfected with pEGFP; Lane2 – sensitive parasites transfected with FesodA-pEGFP. (B) Immunoblot of resistant parasite transfectants' total lysates. Lane 1 - resistant parasites transfected with pEGFP; Lane2 – resistant parasites transfected with FesodA-pEGFP.

4.18 IFA corroborates the transfection and FeSODA depletion in *L. donovani* parasites

IFA was performed to check both the transfection and FeSODA depletion in L. donovani transfectants. The methanol permeabilization allows the primary and secondary antibodies to bind the FeSODA. The GFP fluorescence from the plasmid pEGFP-C1 indicates the presence of the plasmid in the parasites which confirms the successful transfection of the plasmid into the parasites. The AlexaFluor® 594 fluorochrome conjugated to secondary antibody emits red fluorescence with an absorption max. of 590 nm and emission max. of 617 nm. The red fluorescence indicates the expression of FeSODA whereas the absence of red fluorescence indicates the FeSODA depletion. From the following fig., the 1st and 3rd panels indicate the DD8 and BHU875 WT parasites respectively transfected with pEGFP. The 2nd and 4th panels indicate the DD8 and BHU875 KD parasites respectively transfected with pEGFP-antisense FesodA constructs. The GFP fluorescence in all the lanes confirmed that the pEGFP was successfully transfected in all the parasites. The red fluorescence in the 1st and 3rd panels suggested that the FeSODA was expressed in the parasites transfected with pEGFP alone. Whereas the absence of

red fluorescence in 2nd and 4th panels confirmed that the FeSODA was successfully depleted in the parasites transfected with pEGFP-antisense FesodA constructs. Thus, both the transfection and FeSODA depletion were confirmed using IFA.

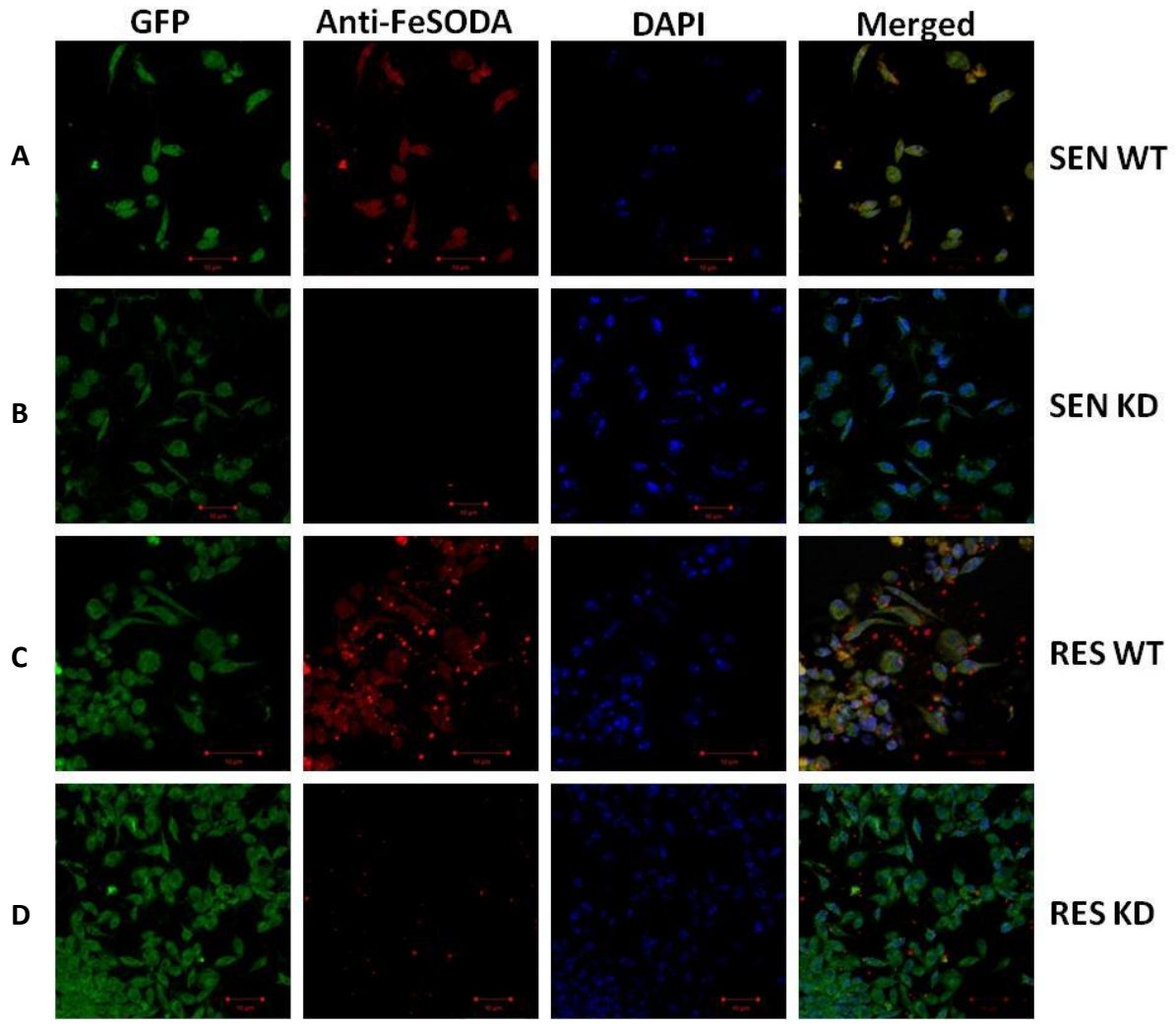


Fig 4.18 Immunofluorescence assay of *L. donovani* transfectants. Panel 1 – GFP fluorescence; Panel 2 - AlexaFluor® 594 fluorochrome fluorescence; Panel 3 – DAPI fluorescence; Panel 4 – Merged. A & D - sensitive and resistant parasites respectively transfected with pEGFP; B & C – sensitive and resistant parasites respectively transfected with FesodA-pEGFP construct.

4.19 FeSODA depletion enhances the susceptibility of resistant parasites to miltefosine

The effect of FeSODA depletion on the miltefosine resistance was assessed by viability test using PI fluorescent dye. PI is a membrane impermeant dye which is generally excluded from viable cells. It binds to the double stranded DNA by intercalating between base pairs. It has an excitation wavelength at 488 nm and emission wavelength at 617 nm. The BHU875 WT and KD parasites were treated with varied concentrations of miltefosine for 48 h. From the fig. below, the parasites transfected with pEGFP alone were able to retain the miltefosine resistance till 60 µM. In contrast, the FesodA knockdown parasites were shown to lose their 50% viability at 20 µM like that of DD8 parasites. This result likely explains that the FeSODA depletion enhances the susceptibility of resistant parasites to miltefosine.

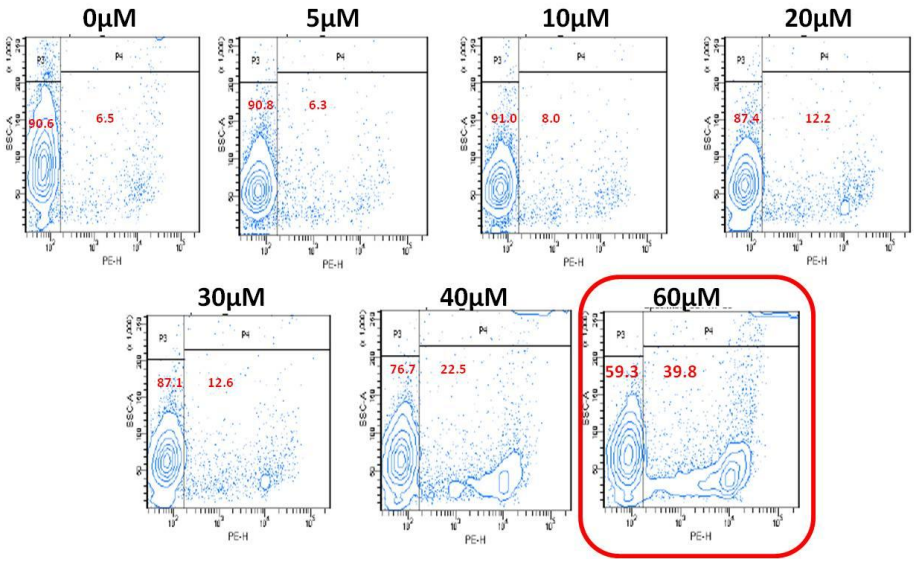


Fig 4.19A Viability assay of resistant parasite-pEGFP transfectants using propidium iodide. The left quadrant indicates the percentage of live cells; the right quadrant indicates the percentage of dead cells. The miltefosine concentrations were indicated on the top of each histogram.

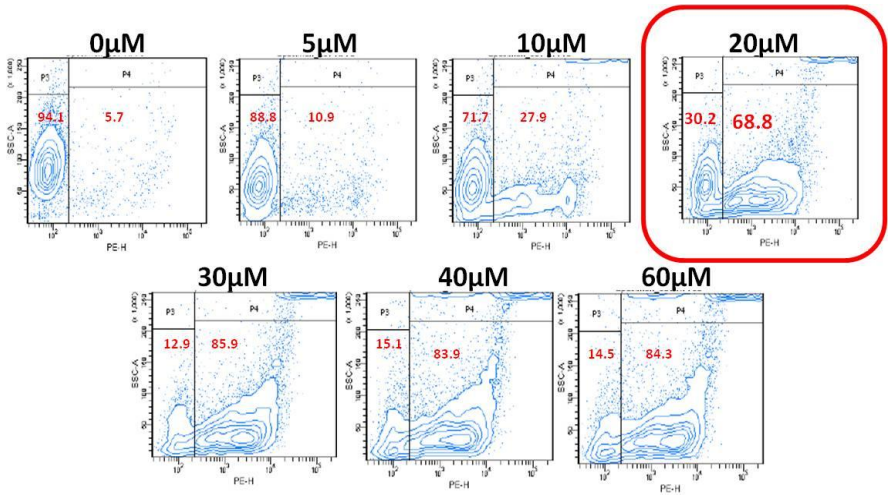


Fig 4.19B Viability assay of resistant parasite-FesodA-pEGFP transfectants using propidium iodide. The left quadrant indicates the percentage of live cells; the right quadrant indicates the percentage of dead cells. The miltefosine concentrations were indicated on the top of each histogram.

4.20 FeSODA depletion reinstates the ROS production in resistant parasites

The ROS levels were estimated in terms of H_2DCFDA mean fluorescence intensity (MFI). H_2DCFDA is a cell-permeant dye and binds to the intracellular ROS. The WT (i.e. transfected with pEGFP) and KD (knockdown i.e. infected with pEGFP-antisenseFesodA) parasites of both the DD8 and BHU875 parasites were treated with 30 μM miltefosine for 24 h, and the ROS was estimated. The MFI of DD8 KD parasite was significantly higher in comparison to that of WT parasites with miltefosine treatment. In case of BHU875 parasites, although the WT parasites exhibited similar MFIs with and without miltefosine treatment, the KD parasites displayed a significant increase in MFI with miltefosine treatment. These results conclude that FeSODA depletion reinstates the ability of miltefosine to produce ROS in the resistant parasites.

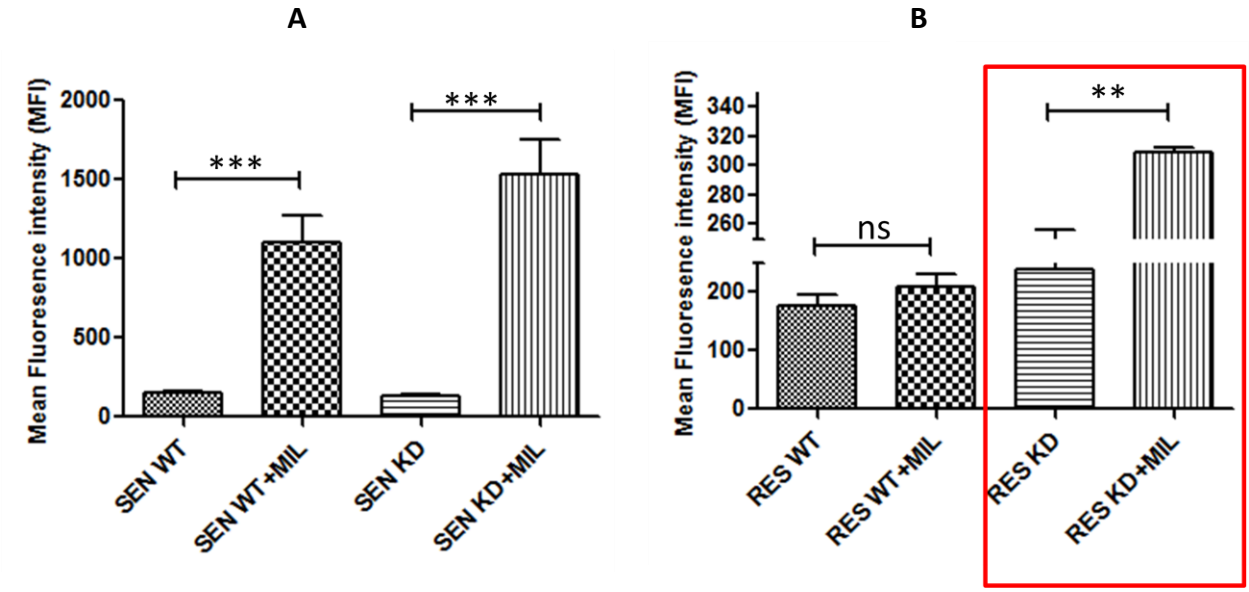


Fig 4.20 ROS estimation in *L. donovani* transfectants with miltefosine treatment. (A) indicates the ROS levels in sensitive parasite WT (containing pEGFP) and KD (containing pEGFP-antisenseFesodA) transfectants. (B) depicts the ROS levels in resistant parasite WT (containing pEGFP) and KD (containing pEGFP-antisenseFesodA) transfectants.

4.21 FeSODA depletion arrests the parasite growth at G2/M phase

The cell cycle of the FesodA knockdown parasites was analyzed by using the PI fluorescent dye. PI binds to all the nucleic acids i.e. both DNA and RNA. Hence, RNase treatment was given to the parasites to avoid the PI binding to RNA. Thus, the fluorescence emits only from the PI binding to DNA that helps in the measurement of the percentage of cells based on their DNA content in different phases of cell cycle. The PI fluorescence is depicted in the following figure. The different bars in the bar graph indicate the different phases of the cell cycle for the time points 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h. The figure explains that the DD8 and BHU875 WT parasites proliferate normally. But the DD8 and BHU875 KD parasites were arrested at G2/M phase of their cell cycle. Thus, this result elucidates that the FeSODA is necessary for the parasite proliferation.

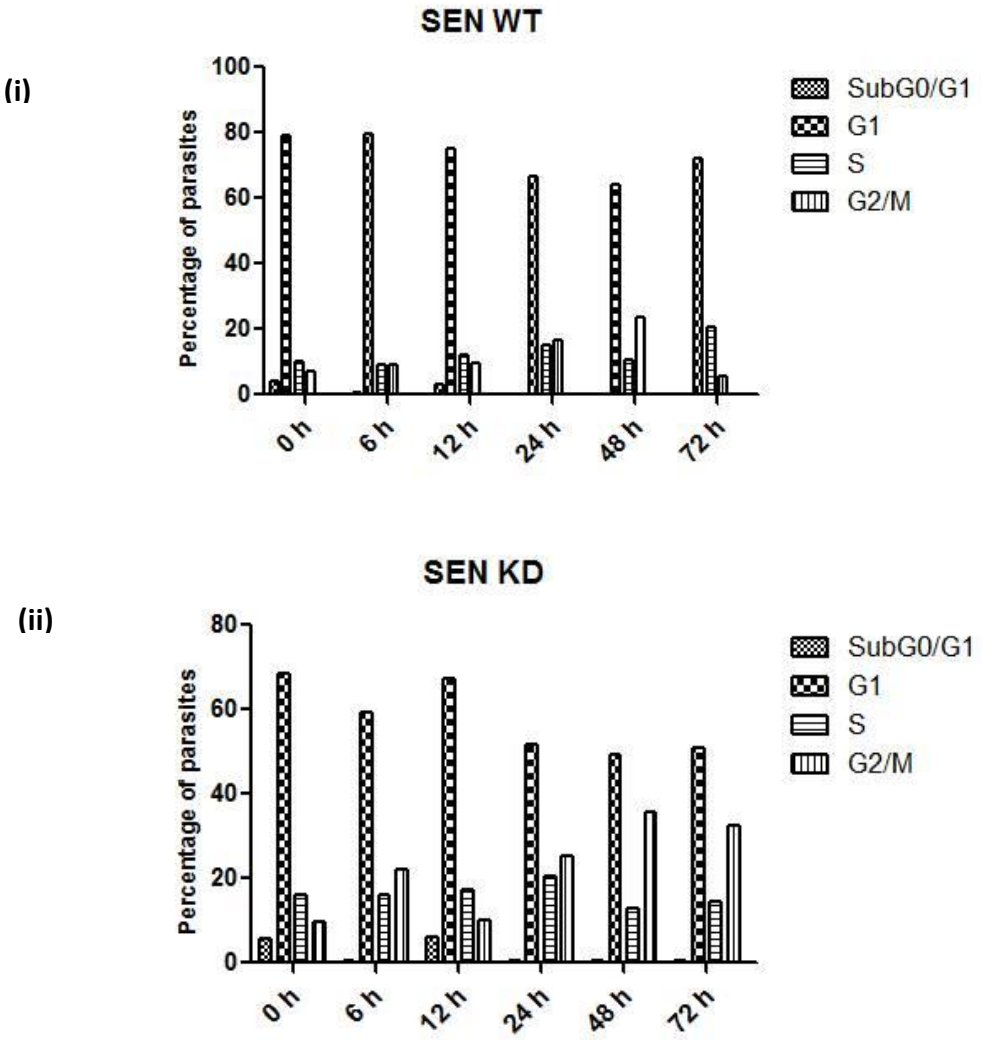


Fig 4.21A Cell cycle analysis of sensitive parasite transfectants. (i) The WT sensitive parasites transfected with pEGFP. (ii) The KD sensitive parasites transfected with pEGFP-antisense FesodA.

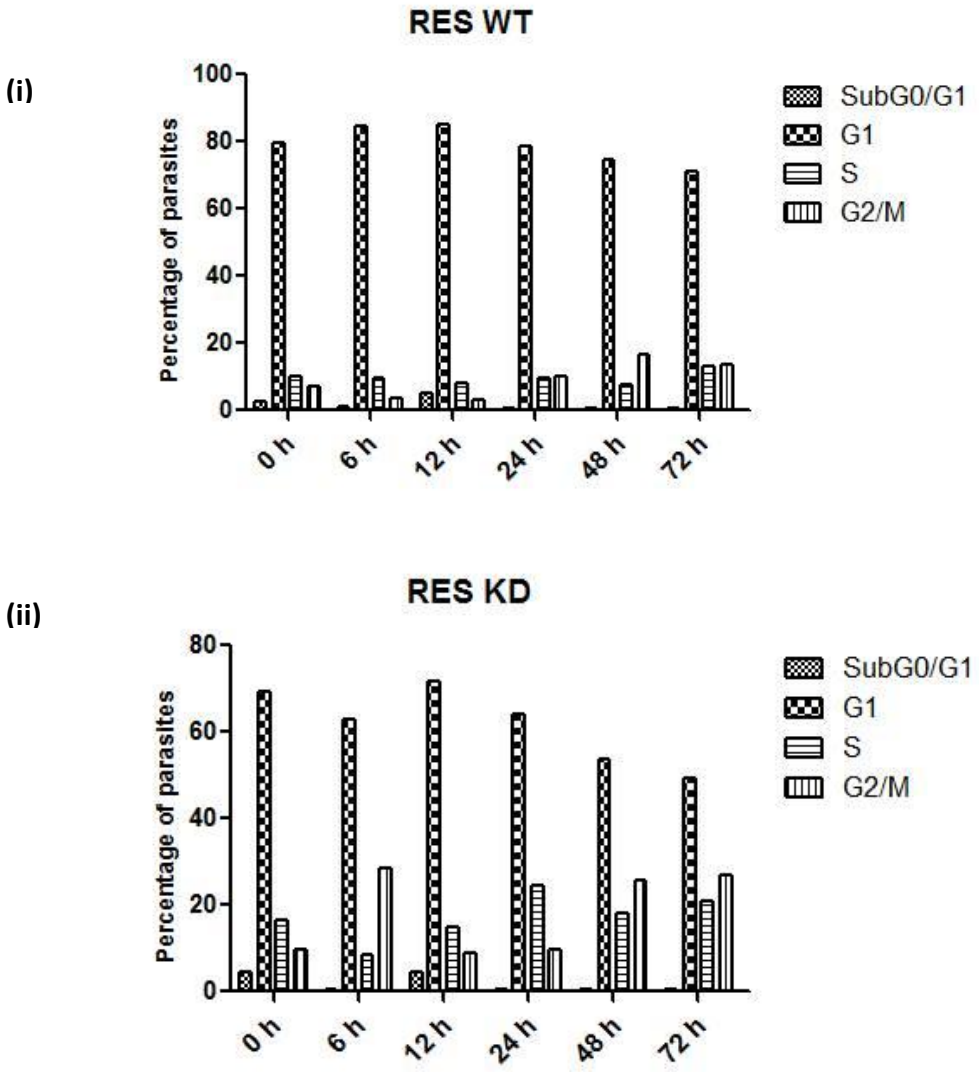


Fig 4.21B Cell cycle analysis of resistant parasite transfectants. (i) The WT resistant parasites transfected with pEGFP. (ii) The KD resistant parasites transfected with pEGFP-antisense FesodA.

4.22 FeSODA depleted parasites do not manifest any change in morphology

The morphology of the transfectants was observed microscopically by Giemsa staining. DD8 and BHU875 WT and KD parasites were stained with Giemsa for about 20 min. The microscopical observation clarified that both the WT and KD parasites exhibited similar morphology i.e. slender and elongated.

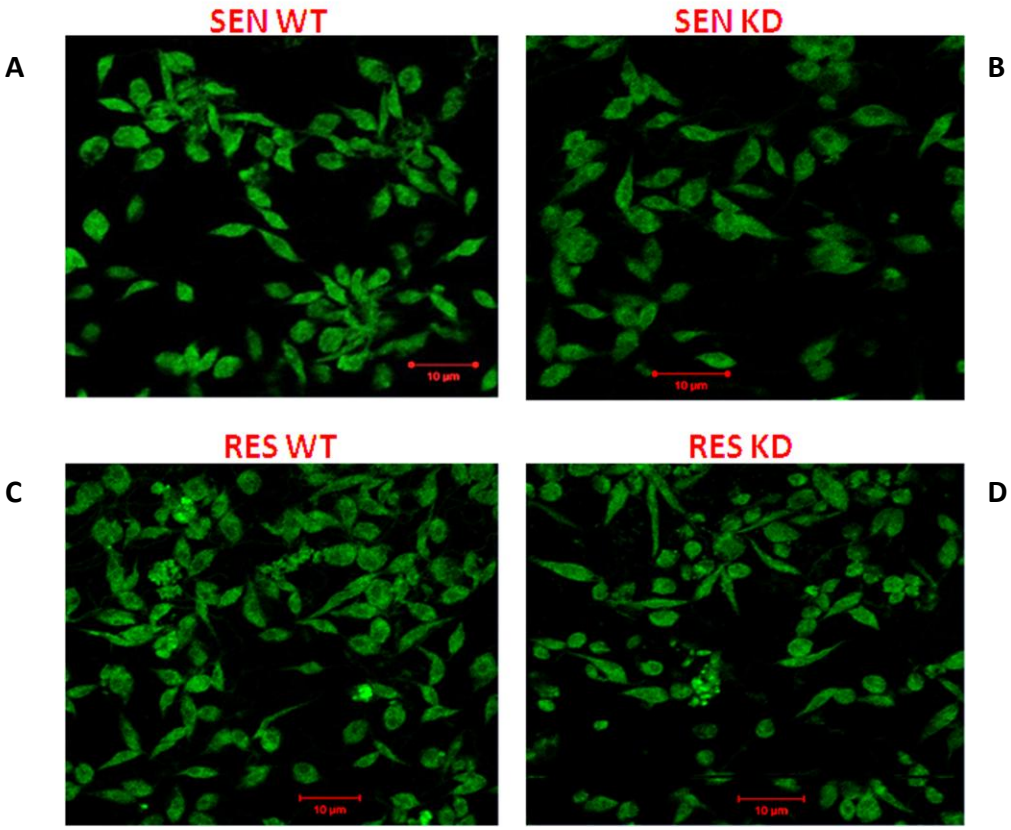


Fig 4.22 Morphology of *L. donovani* transfectants by confocal microscopy. A- Sensitive parasites transfected with pEGFP; B – Sensitive parasites transfected with pEGFP-antisenseFesodA; C - Resistant parasites transfected with pEGFP; D – Resistant parasites transfected with pEGFP-antisenseFesodA

4.23 pEGFP plasmid is stable in the intracellular parasites

The stability of fluorescence in the macrophages was measured by confocal microscopy. The macrophages were infected with DD8 and BHU875 WT and KD parasites for 72 h. The confocal microscopy showed the emission of fluorescence in all the parasites. This suggests that the pEGFP plasmid is stable in the intracellular parasites.

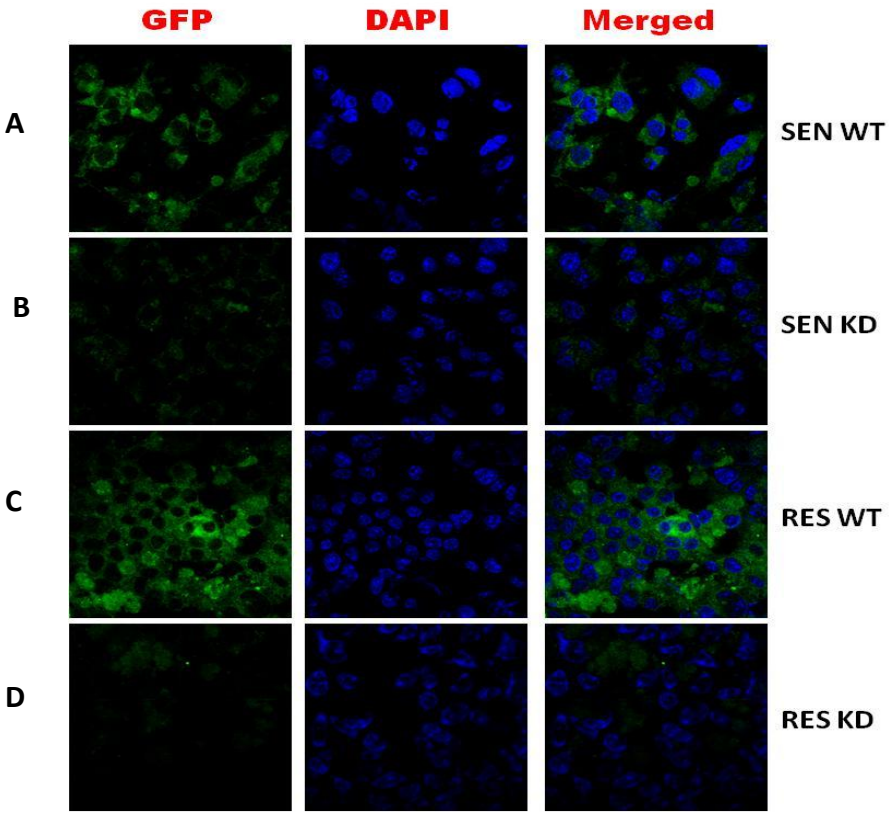


Fig 4.23 Monitoring the stability of pEGFP plasmid inside the macrophages after 72 h. The green fluorescence in all the panels indicate that the pEGFP stable in the macrophages for 72 h. Panel A – Sensitive parasites with pEGFP; B – Sensitive parasites with pEGFP-antisense FesodA; C – Resistant parasites transfected with pEGFP; D – Resistant parasites transfected with pEGFP-antisenseFesodA

4.24 Depletion of FeSODA affects the parasite replication inside the macrophages

The survival of the transfectants was estimated from the counting of a number of amastigotes microscopically. The infected macrophages were stained with Giemsa stain, and the amastigotes were counted per 100 macrophages. The amastigotes number was depicted in the bar graphs below. The graph clearly points out that the number of DD8 KD amastigotes significantly reduced than that of DD8 WT amastigotes. The BHU875 KD amastigotes number was also significantly reduced when compared to the WT amastigotes number. This likely explains that

FeSODA is essential for the intracellular parasite replication. The miltefosine treatment also significantly reduced the BHU875 KD amastigotes number which points toward the loss of resistance in the intracellular KD parasites.

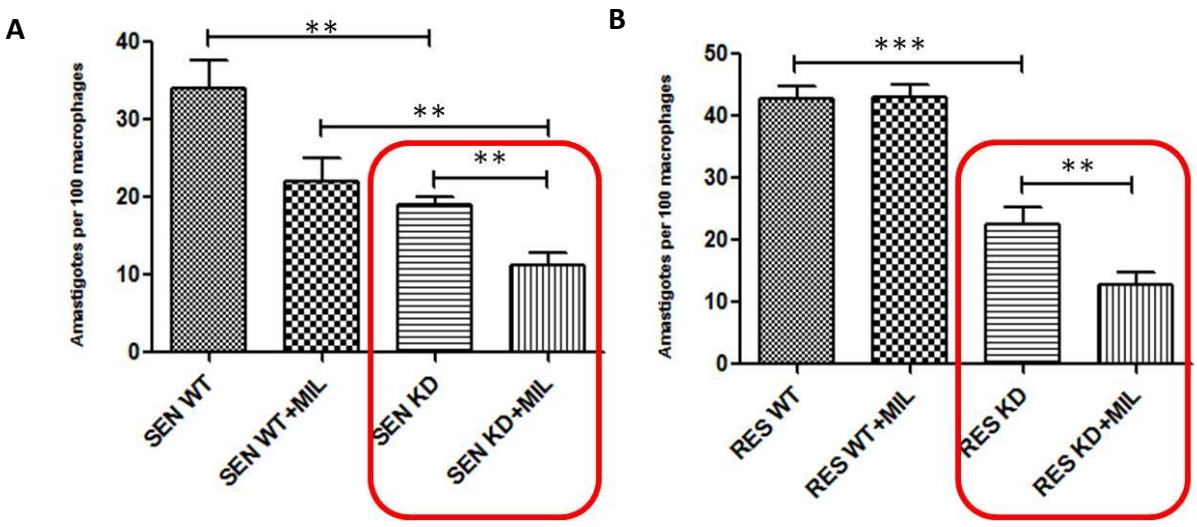


Fig 4.24 Amastigote counting in J774A.1 macrophages. (A) The macrophages infected with sensitive knockdown parasites and miltefosine treated macrophages infected with wild type sensitive parasites. (B) The macrophages infected with resistant knockdown parasites and miltefosine treated macrophages infected with wild type resistant parasites.

4.25 FeSODA plays a dispensable role in the parasite infectivity

The infectivity of the intracellular parasites was assessed by confocal microscopy. The GFP fluorescence is directly proportional to the parasite infectivity. The DD8 and BHU875 WT and KD parasites were analyzed for GFP fluorescence after 6 h infection. The confocal microscopy revealed that the GFP fluorescence was almost similar in all the parasites indicating that both the WT and KD parasites are able to infect the macrophages. This symbolizes that the FeSODA is not necessary for the infectivity of the parasites.

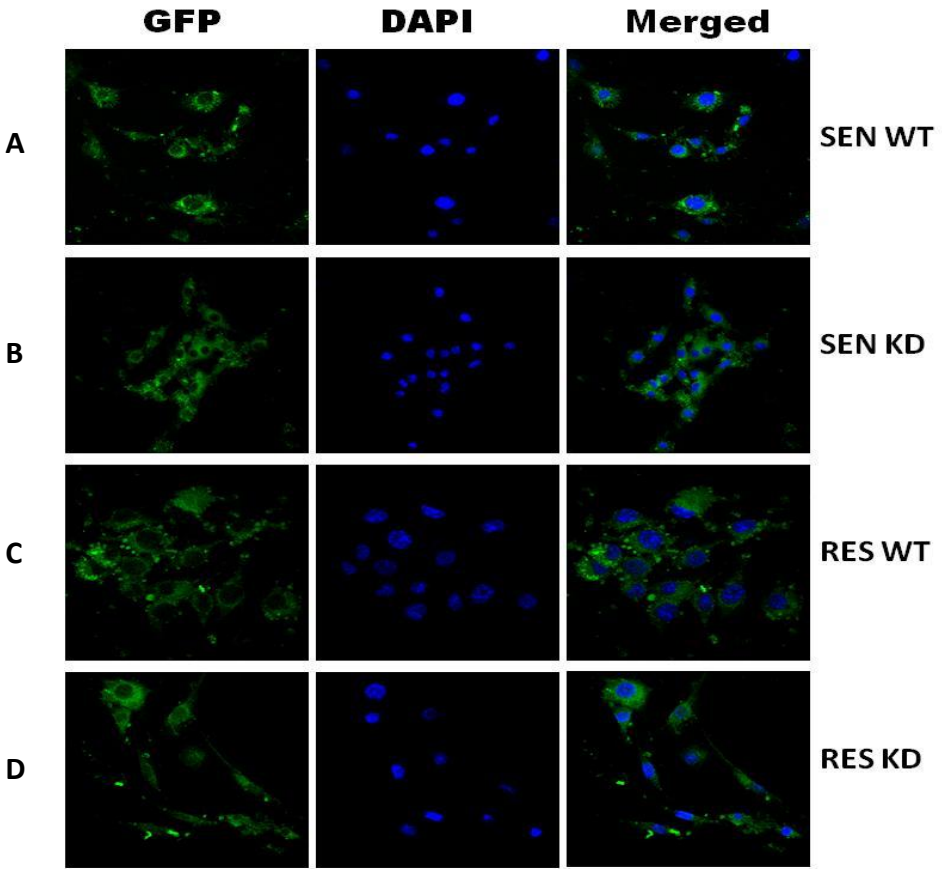


Fig 4.25 Monitoring of green fluorescence in J774A.1 macrophages infected with *L. donovani* transfectants. (A) Macrophages infected with sensitive-pEGFP transfectants; (B) Macrophages infected with sensitive knockdown transfectants; (C) Macrophages infected with resistant-pEGFP transfectants; (D) Macrophages infected with resistant knockdown transfectants.

4.26 LC-MS/MS analysis of host macrophage proteins

The differentially expressed proteins during infection with sensitive and resistant parasites were analyzed using LC-MS/MS approach. Using the WebGEStalt software, the proteins were analyzed based on their cellular compartment and pathways. The mitochondrial proteins and the pathways upregulated in the macrophages infected with resistant parasites are listed below.

S. No.	Protein	Function
1	Beta-carotene oxygenase 2	Protects against carotenoid-induced mitochondrial dysfunction and ROS
2	Mitochondrial ribosomal protein L19	Helps in protein synthesis
3	Aminolevulinic acid synthase 1	Heme biosynthesis
4	Benzodiazepine receptor associated protein 1	Binds with PBR which mediates the intramitochondrial cholesterol transport, a rate-limiting step in steroid biosynthesis
5	MLX interacting protein	Involved in glucose metabolism and lipid synthesis
6	RIKEN cDNA 2510003E04 gene (KIF1bp)	Required for proper distribution of mitochondria in cell
7	Malate dehydrogenase 2, NAD	Increases ATP levels and decreases ROS production
8	Solute carrier family 22, member 21	Transports carnitine essential for beta-oxidation
9	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	Involved in the synthesis of guanidinoacetic acid and ornithine
10	Nardilysin, N-arginine dibasic convertase, NRD convertase 1	Mediates cell proliferation and migration
11	RD RNA-binding protein	Binds to Early growth response 1 (Egr1) which might inhibit Stat3
12	Farnesyl diphosphate synthetase	Cholesterol, sterol, heme A synthesis
13	Cytokine induced apoptosis inhibitor 1	Has anti-apoptotic effects in the cell
14	Beclin 2	Anti-apoptotic

Table 4.2 List of mitochondrial proteins upregulated in the macrophages infected with miltefosine resistant parasites

S. No.	Pathways	No. of genes	Enrichment analysis
1	Phosphatidylinositol signaling system	6	KEGG pathways
2	Inositol phosphate metabolism	5	KEGG pathways
3	Phosphatidylinositol phosphorylation	5	GO analysis
4	Glycine, serine and threonine metabolism	3	KEGG pathways
5	Urea cycle and metabolism of amino groups	2	Wiki pathway

Table 4.3 List of the pathways differentially regulated in the macrophages infected with miltefosine resistant parasites

S. No.	Protein	Function
1	Synaptojanin 2 (**SYNJ2**)	Effector of Rac1 and helps in cell proliferation (*Malecz N et al., 2000*)
2	Phosphoinositide kinase, FYVE finger containing(**PIKfyve**)	Regulates actin cytoskeleton (*Assia Shisheva, 2008*) and helps in phagosome maturation (*Kim GH et al., 2014*)
3	Inositol polyphosphate-5-phosphatase D (**SHIP1**)	Decreases NF-κB activation and also inhibits TLR signaling (*Conde C et al., 2012)*
4	Phosphatidylinositol 3-kinase, C2 domain containing, alpha polypeptide (**PIK3C2A**)	Induces proliferation of clathrin-coated structures in the cytoplasm (*Zhao Y et al., 2007*)
5	Phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide (**PIK3C2G**)	Promotes cell survival
6	Inositol polyphosphate-4-phosphatase, type I (**INPP4A**)	Involved in transferrin internalization (*Shin et al., 2005*)

Table 4.4 List of the proteins upregulated in phosphatidylinositol signaling system

S. No.	Protein	Function
1	Aminolevulinic acid synthase 1	Heme biosynthesis (*Hunter GA and Ferreira GC, 2011*)
2	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	Produces creatine which downregulates TLR2, TLR3 and also produces ornithine (Bera S et al., *2008*)
3	Pipecolic acid oxidase	Metabolizes sarcosine to glycine

Table 4.5 List of the proteins upregulated in Glycine, serine and threonine metabolism

Chapter 5:

DISCUSSION

In the present scenario, miltefosine is being used as the first line of treatment for treating visceral leishmaniasis. The major bottleneck in the treatment of VL is the emergence of resistance in *Leishmania* parasites against the currently available drugs. No effective vaccine for the treatment of leishmaniasis is available hitherto. Miltefosine, the only oral drug, was found to be effective but some patients have been found to show relapse against this drug. This is likely because of the higher half-life (150-200 h) in the blood plasma. The *Leishmania* parasite is tricky enough to evolve the mechanisms that overcome the drug actions and help in its survival in the phagosomal niche. These evolved resistant mechanisms impede the current chemotherapy in the treatment or eradication of leishmaniasis. As the parasite is able to acquire resistance against almost all the currently available drugs, one of the possible remedies that could ameliorate the present situation is to understand the mechanism of resistance acquired by the parasites. Unraveling the resistance mechanism could pave a way for the future research towards the development of vaccine/drug that is able to dodge the parasite's ability of drug resistance. Exploitation of proteomics approach to analyze the differentially regulated proteins in both the sensitive and resistant parasites could provide inkling to the development of resistance mechanism.

Several drugs have been used in the chemotherapy for the treatment of VL viz. sodium stibogluconate, amphotericin B, and paromomycin. Previous reports made an attempt to study the differentially regulated proteins in different drug resistant strains i.e. sodium stibogluconate, amphotericin B, paramomycin, etc. (*Kumar, Sisodia et al. 2010*; *Chawla, Jhingran et al. 2011*; *Brotherton, Bourassa et al. 2014*). For the first time, our current study focuses on the differential proteomics in miltefosine resistant strain. Among the drugs being used, miltefosine has been shown to be very effective in the clinical trials and also it being the first oral drug. So, our present study was carried out using clinical miltefosine resistant strain, BHU875, and DD8 (WHO reference strain), of *L. donovani* parasites. The miltefosine drug toxicity against the parasite was determined by the cytotoxicity assay and was found to be ~80±5 μM for resistant parasites whereas the sensitive parasites were found to be susceptible to ~15±5 μM miltefosine concentration. Analyzing their cell cycle and ROS MFIs with the miltefosine treatment has further validated the resistance in BHU875 strain. The down regulated IFN-γ mRNA levels in resistant strain likely suggests that the parasite is down modulating the IFN-γ mediated immune

response mediated by miltefosine. The NO estimation also suggests that the BHU875 is able to overcome the NO production by miltefosine *in vitro*. This indicates that the resistance is being maintained in the intracellular amastigote stage also.

It is previously reported from various studies that the 94% of the *Leishmania* genome is constitutively expressed in both the stages (*Leifso, Cohen-Freue et al. 2007*). The isolation of amastigotes from macrophages is tedious, needs expertise and their maintenance *in vitro* is difficult. Therefore, we have studied the differential protein expression at the promastigote stage. Hence, we approached 2D-gel electrophoresis to analyze the differentially regulated proteins in the resistant parasites compared to the sensitive parasites. From our study, we could find 12 differentially regulated proteins among which 4 proteins were upregulated and 8 were down regulated. The upregulated proteins include a hypothetical protein, iron superoxide dismutase, metallopeptidase M16 and metallopeptidase M32. The iron superoxide dismutase protein helps in the dismutation of superoxide radicals and protects the parasite from oxidative killing and favors the parasite survival. And both the metallopeptidases belonging to the families M16 and M32 have a metallopeptidase activity. The down regulated proteins comprise two hypothetical proteins with an unknown function, phosphomannomutase involved in mannose metabolism, 3-ketoacyl co-A thiolase like protein involved in fatty acid beta oxidation, tryparedoxin, enolase playing a role in glucose metabolism and ubiquitin fusion protein which is a constituent of ribosome. Thus, our proteomics study at promastigote stage provides a diminutive clue for proteins that are differentially expressed in miltefosine resistant BHU875 strain. However, further detailed study has to be undertaken to decode the definite resistance mechanism acquired by the parasite against miltefosine drug.

From the validation experiments, we have observed that the miltefosine was able to produce ROS in case of sensitive parasites when treated with miltefosine for 24 h. But when the resistant parasites were treated with miltefosine, it is likely that either the resistant parasites were able to surmount the ROS production by miltefosine or the miltefosine was unable to produce ROS. Our proteomics analysis of resistant parasites revealed the up regulation of their FeSODA, which controls the superoxide radicals produced during the oxidative killing. It likely suggests that the up regulated FeSODA helps in combating the ROS effect produced by miltefosine in the

resistant parasites. To unfasten this knot, we have approached the episomal inhibition of FeSODA using the antisense FesodA construct of pEGFP-C1 mammalian expression vector. The GFP encoded by the EGFP gene was used for the confirmation of the transfection and the G418 drug against the neomycin was used for the selection of transfectants. The antibody was also raised in BALB/c mice against *L. donovani*'s FeSODA. The difference in the expression levels in FeSODA (i.e. 1.5 fold increase in resistant strain) confirms the overexpression of FeSODA in the resistant parasites. The IFA confirms the transfection and also the FeSODA inhibition in both the sensitive and resistant parasites. The enhancement in the susceptibility of FeSODA resistant parasites towards miltefosine signifies that the FeSODA protein is essential for the determination of parasite susceptibility (or resistance) towards miltefosine. The ROS production in FeSODA depleted parasites symbolizes that the FeSODA levels plays a critical role in their susceptibility towards miltefosine through its suppressive effect on ROS. Cell cycle arrest at G2/M phase of FeSODA depleted parasites also provides a clue that the FeSODA plays a role in the cell cycle progression of the parasites. But the morphology of the parasites was not affected indicating that the FeSODA depletion has no role in the morphology. The stability of the episomal plasmid in the intracellular macrophages is not clear. In our study, the fluorescence microscopic analysis of the infected macrophages clarified that the episomal plasmid is stable for 72 h. In this process, a decreased fluorescence was observed in the case of knockdown parasites' infected macrophages. The decreased knockdown amastigote count in the macrophages showed that the FeSODA is indispensable for the intracellular parasite survival. But the fluorescence after 6 h suggested that FeSODA is dispensable for the parasite infectivity. Our study shows that the FeSODA could be used as a target for chemotherapy; the combinatorial effect of miltefosine with FeSODA targeting drugs could help in increasing the effectivity of miltefosine and also might contribute to overcome the miltefosine resistance.

Previous report (*Ghosh, Goswami et al. 2003*) has showed the iron superoxide dismutase depletion in *L. donovani* with a part of *L. tropica* gene sequence. But, in this case, there is a possibility of inhibition of two more putative sequences (LdBPK_321910.1 and LdBPK_321920.1) present in *L. donovani*. Hence, it cannot explain the role of any specific gene in the survival of *Leishmania*. So, our antigene approach of inhibition targets only the FesodA

(LdBPK_080300.1) using its whole gene sequence in an antisense orientation. In addition, our study was attempted to understand the role of FeSODA in the miltefosine resistance. Our finding of FeSODA overexpression in miltefosine resistant parasites is in accordance with the previous report showing overexpression of FeSODA protects the parasites from *L. donovani* from the miltefosine mediated programmed cell death (*Getachew and Gedamu 2012*).

The differential proteomics of the drug sensitive and resistant parasites could provide the traces of resistance mechanism that has been acquired by the parasites. However, the fact that the host molecular mechanisms are modulated during the parasite infection cannot be excluded. In addition to the modulation in the parasite signaling molecules, there is a sheer possibility of modulation in host signals by the parasite. Hence, in order to attain the goal of understanding the resistance mechanism, it is quite important to study the differential proteomics of the host macrophages too. The J774A.1 murine macrophage proteins were considered for this study. These macrophage proteins were isolated 24 h post-infection and subjected to label-free LC-MS/MS quantification to investigate the modulations in the early events of macrophages. The label-free quantification does not use isotope labeling. Hence, each sample is measured in a separate LC-MS/MS experiment. It also provides the advantage of no sample loss and no introduction of errors that are usually faced in labeling experiments.

In our study, label-free LC-MS/MS was performed using nano Acquity Waters UPLC system. From the proteins identified, we have considered the upregulated proteins in the macrophages infected with miltefosine resistant parasites.

The identified proteins in both the macrophages infected with the sensitive as well as resistant parasites were analyzed by using WebGEStalt online program which uses GO analysis, KEGG pathway analysis and Wikipathway analysis. For the analysis, significance level of $p<0.01$ was used. The proteins were first analyzed based on the cellular compartments in which they are expressed. About 14 mitochondrial proteins were found to be upregulated in the macrophages infected with resistant parasites. These include Beta-Carotene Dioxygenase 2 (BCDO2), Mitochondrial ribosomal protein L19, 5'-Aminolevulinate synthase 1 (ALAS1), Benzodiazepine receptor associated protein 1 (BZRAP1), MLX interacting protein (MLXIP), KIF1bp, Malate

dehydrogenase 2 (MDH2), Solute carrier family 22 member 21 (Slc22a21), Glycine amidinotransferase (GATM), Nardilysin (NRD1), RD RNA binding-protein (RDRBP), Farnesyl diphosphate synthetase (FPPS), Cytokin- induced apoptosis inhibitor 1 (CIAPIN1), and Beclin 2 (Bcl2).

BCDO2 is a mitochondrial carotenoid oxygenase. Carotenoids are the precursor of vitamin A. BCDO2 maintains the carotenoid homeostasis in the cells. The accumulated carotenoids can impair mitochondrial respiration and induce oxidative stress which leads to the release of cytochrome c, proteolytic activation of caspase 3 and PARP1 resulting in apoptosis. BCDO2 scavenges and degrades the carotenoids in turn acting as a gatekeeper for the intrinsic apoptosis (*Amengual, Lobo et al. 2011; Lobo, Isken et al. 2012*). Hence, the overexpression of BCDO2 likely suggests that the resistant parasites might be suppressing the miltefosine induced apoptosis through BCDO2.

ALAS1 is involved in the shemin pathway which occurs in mitochondria. It catalyzes the condensation of glycine and succinyl-CoA to aminolevulinic acid (*Soldati 2007*). It is the first and also the rate-limiting enzyme in the heme biosynthesis (*Sassa 1988*). It provides heme for cytochromes and hemeproteins (*Furuyama, Kaneko et al. 2007*). The heme regulates it through the negative feeback mechanism *(Schuurmans, Hoffmann et al. 2001; Furuyama, Kaneko et al. 2007*). The overexpression of ALAS1 might result in the excess heme synthesis during the parasite infection as parasite utilizes host's heme for its survival inside the macrophages. This regulation is mediated by Egr-1 (Early growth response 1) and NAB1/2 complex binding to the HRE (heme-responsive element) region in ALAS1 mRNA. Thus, the heme synthesis is regulated (*Gotoh, Nakamura et al. 2011*). In addition to ALAS1, the BHU875 infected macrophages also shown to express RD RNA-binding protein. This protein binds to Egr-1 and negatively regulates Egr-1. Hence, the binding of Egr-1 to ALAS1 is inhibited by RD RNA-binding protein and the heme synthesis is uninterrupted even in its excess conditions. Thus, the resistant parasites might be exploiting the macrophages through this process for its heme dependence. Besides the negative regulation of ALAS1, Egr-1 is also known to inhibit the Stat3 signaling. Stat3 is also necessary for the intracellular parasite proliferation as it triggers IL-10 production which is essential for the parasite persistence in the macrophages.

BZRAP1 binds to the PBR, a mitochondrial protein (*Galiègue, Jbilo et al. 199*9). It regulates the cholesterol transport from the outer to the inner mitochondrial membrane, a rate-limiting step in steroid biosynthesis (*Papadopoulos 2004*). This might help in the excess production of steroids during the parasite infection. MLXIP (MLX interacting protein) is also known as MondoA. It forms a heterodimer with MLX (Max-like protein X) and binds to the DNA. It is known to play a role in the transcriptional activation of glycolytic target genes (*Sans, Satterwhite et al. 2006*). It regulates the genes in response to the cellular glucose levels (*Peterson, Stoltzman et al. 2010*). MondoA:MLX complex is localized to the outer mitochondrial membrane and shuttles between the mitochondria and the nucleus (*Eilers, Sundwall et al. 2002*). This complex senses and monitors the intracellular glucose-6-phosphate levels. When the G6P metabolite levels are high, the MondoA:MLX complex enters the nucleus wherein binds to the thioredoxin-interacting protein (TXNIP) and negatively regulates the glucose uptake (*Stoltzman, Peterson et al. 2008*). Though the TXNIP is known to mediate ROS-dependent inflammasome activation *(Zhou, Tardivel et al. 2010*), a study on *Leishmania* GP63 metalloprotease revealed that GP63 cleaves the TXNIP and suppresses the NLRP3 inflammasome-mediated IL-1β production *(Shio, Christian et al. 2015*). This likely shows that the elevated glucose levels during the infection with intracellular resistant parasites are controlled by MondoA:MLX complex whereas the elevated TXNIP levels during this process in turn cleaved by *Leishmania* GP63. Thus, the resistant parasites maintain a balance in the cellular homeostasis to promote their survival inside the macrophages.

KIF1bp is required for the proper distribution of mitochondria in the cell *(Wozniak, Melzer et al. 2005*) thus helping in the proper functioning of the mitochondria during the infection with the resistant parasites. MDH2 is the enzyme participates in TCA cycle. It converts malate/NAD^+ to oxaloacetate/NADH. This NADH is the substrate for oxidative phosphorylation and the ATP is synthesized (*Goward and Nicholls 1994; Reisch and Elpeleg 2007*). The overexpression of MDH2 likely suggests that the ATP levels increases enhancing the mitochondrial energy metabolism. In the mitochondria producing high levels of ATP and the lower NADH/NAD^+, the ROS production is lower (*Murphy 2009*). In addition, a study on the knockdown of MDH2 in prostate cancer cell showed that there is a disruption in the final step of TCA cycle, decrease in

ATP production and increase in ROS formation (*Liu, Harvey et al. 201*3). This also provides a clue that the overexpression of MDH2 might help in protecting from ROS production during the infection with resistant parasites.

Slc22a21, also known as OCTN3 and Slc22a9, is a carnitine transporter. Carnitine is essential for the mitochondrial oxidation of long-chain fatty acids and ATP generation (*Bremer 1983*). It decreases oxidative stress, inhibits apoptosis and caspases, and also decreases inflammatory cytokines (*Pastorino, Snyder et al. 1993*; *Cifone, Alesse et al. 1997*; *Mutomba, Yuan et al. 2000*). GATM or AGAT catalyzes the transamidation of guanidine group from arginine to glycine, yielding guanidinoacetic acid (GAA) and ornithine (*Gross, Eggen et al. 1986*). Ornithine is involved in polyamine, glutamine, and proline synthesis. It is also a rate-limiting enzyme in creatine biosynthesis (*Humm, Fritsche et al. 1997*). Nardilysin is a metallopeptidase belonging to the M16 family. It specifically cleaves the basic residues and is essential for cell migration and proliferation (*Prat 2004*). Its activity is increased by malate dehydrogenase. NRD1 facilitates the complex formation between malate dehydrogenase (MDH) and citrate synthase (CS). The MDH and CS complex usually form a complex called metabolon and increases the metabolic efficiency (*Morgunov and Srere 1998*). Thus, the overexpression of nardilysin might help in boosting the cellular energy metabolism during the infection with resistant parasites.

Farnesyl diphosphate synthase (FPPS) is an essential enzyme in the isoprenoid bisoynthesis pathway. It catalyzes the farnesyl pyrophosphate (FPP) biosynthesis (*Poulter and Rilling 1981*). FPP is the precursor of several products namely, sterols, dolichols, the isoprenoid moieties of ubiquinone, heme a, and prenylated proteins. CIAPIN1, or Anamorsin, is an anti-apoptotic molecule (*Shibayama, Takai et al. 2004*). The overexpression of CIAPIN1 might be conferring resistance to apoptosis in the macrophages infected with resistant parasites. A study on leukemia cells showed that the overexpression of CIAPIN1 upregulates the expression of MDR-1 and Bcl-2 (*Li, Hong et al. 2007*). In compliance with this report, we could also found the overexpression of Bcl-2 anti-apoptotic factor. This likely suggests that the overexpression of CIAPIN1 and Bcl-2 promotes the cell survival by conferring resistance to apoptosis.

Thus, the overexpressed mitochondrial proteins likely indicate that the resistant parasites are able to modulate the host cell mechanisms by suppressing the apoptosis. It enhances the cholesterol and heme synthesis essential for the parasite survival inside the macrophages. It also increases the host cellular energy metabolism.

The differentially regulated biological pathways were also analyzed using the WebGEStalt online program. From our analysis, we could find that the phosphatidylinositol signaling system, inositol phosphate metabolism, phosphatidylinositol phosphorylation, glycine-serine-threonine metabolism, urea cycle and metabolism of amino groups were found to be modulated in the macrophages infected with resistant parasites.

The proteins involved in the glycine-serine-threonine metabolism, urea cycle and metabolism of amino groups i.e. GATM and ALAS1 were discussed previously. The proteins involved in the remaining pathways include SYNJ2, PIKfyve, SHIP1, PIK3C2A, PIK3C2G and INPP4A. SYNJ2 (synaptojanin 2) is a polyphosphoinositide phosphatase. It dephosphorylates PI(3,4,5)P2 to PI(3,4)P2. In addition to its role in PI metabolism, it also acts as a Rac1 effector in a GTP-dependent manner. It is translocated to the plasma membrane by Rac1 and together inhibits the clathrin-mediated endocytosis of epidermal growth factor (EGF) and transferrin receptors (*Malecz, McCabe et al. 2000*). This is important for the regulation of cell proliferation. PIKfyve binds to the PI3P and PI at the membrane, phosphorylates them at D-5 position and synthesizes PI(3,5)P2 and PI5P respectively (*Sbrissa, Ikonomov et al. 1999*). It is associated with early endosomes and regulates the retrograde endosome-trans golgi network transport *(Rutherford, Traer et al. 2006*). It regulates endomembrane homeostasis and F-actin remodeling *(Shisheva 2008*). It also participates in the phagosomes maturation (*Kim, Dayam et al. 2014*).

SHIP-1, or Inpp5d, is an inositol 5'-phosphatase containing SH2- domain. It hydrolyzes PI(3,4,5)P2 and synthesizes PI(3,4)P2. It negatively regulates the immune pathways *(Condé, Gloire et al. 2011*). It is known to downmodulate the TLR signaling *(An, Xu et al. 2005; Gabhann, Higgs et al. 2010*). It decreases NF-κB activation by interacting with XIAP and perturbing the association of XIAP and RIP2 required for the NF-κB activation (*Conde, Rambout*

et al. 2012). SHIP-1 was also reported to downregulate IL-12 production by macrophages and elp in the development of Th2 immune response (*Hadidi, Antignano et al. 2012*).

PIK3C2A is required for the activation of Hedgehog (Hh) pathway thereby resulting in the increase of angiogenic factors, cyclins, anti-apoptotic genes, and decrease in apoptotic genes (Franco, Gulluni et al. 2014); (Lee, Moskowitz et al. 2007); (Adolphe, Hetherington et al. 2006); (Athar, Li et al. 2004)). The abnormal activation of Hh pathway has been observed in the case of cancers (*Xie, Murone et al. 1998; Rubin and de Sauvage 2006*). But the role of its abnormal activation in case of VL has to be determined. It is required for dynamin-independent endocytosis, vascular endothelial growth factor (VEGFR2), and sphingosine-1-phosphate ($S1P_1$) receptors (Krag, Malmberg et al. 2010); (Yoshioka, Yoshida et al. 2012); (Biswas, Yoshioka et al. 2013). The exact function of the protein PIK3C2G is not yet determined. It synthesizes PI3P and PI(3,4)P2 secondary messengers. INPP4A, a type I inositol polyphosphate 4-phosphatase, removes the phosphate group at 4^{th} position of inositol 3,4-bisphosphate. It plays a role in controlling normal endosome function and also in transferrin internalization *(Ivetac, Munday et al. 2005; Shin, Hayashi et al. 2005*).

Thus, these above discussed proteins regulate other cellular functions in addition to the phosphatidylinositol signaling system. These proteins' expression levels are modulated such that their functions help in the host cell proliferation, survival and also in turn help in the intracellular resistant parasite survival. The resistant parasites successfully establish the infection and alter host's energy metabolism, suppresses reactive oxygen species, enhances the synthesis of molecules necessary for their intracellular survival, downmodulate the defensive immune response for their persistence inside the host macrophages. Our study provides a clue for the proteins that are altered in the host macrophages during the infection with miltefosine resistant parasites. Further elaborative studies are required to gain an insight into the molecular mechanisms developed by the resistant parasites that help them in outdoing the miltefosine action against them.

Chapter 6:

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Role of leptin in human visceral leishmaniasis? ☆

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ABSTRACT

Human visceral leishmaniasis (VL) is frequently found in poor population who are suffering from malnutrition in endemic areas. Therefore, obviously they may have reduced levels of leptin due to reduction in number of adipocytes which are major source of leptin production. Human pathogenesis of VL and reduced levels of leptin both are associated with increase in Th2 type immune response, characterized by secretion of cytokines such as IL-4 and IL-10. Whereas, the protective immune response during visceral leishmaniasis is associated with effective Th1 type immune response characterized by secretion of IFN-γ, IL-2 and IL-12, which correlates with leptin induction of T cells polarizing to Th1 population and secretion of proinflammatory cytokines, and also inhibition of Th2 type response. Therefore, we hypothesized that leptin might be effective in treatment of visceral leishmaniasis alone or VL patients who have co-infection with other immune deficiency syndromes such as AIDS/diabetes/autoimmune disorders by regulation of Th1/Th2 homeostasis.

Introduction

Kala-azar or human visceral leishmaniasis (HVL) is caused by protozoa belongs to the genus *Leishmania* which infect host macrophages. In India, *L. donovani* is responsible for this disease which is characterized by parasite invasion of lymphoid tissues without causing clinical symptoms [16]. The disease is spread by the bite of certain species of sand fly (subfamily–Phlebotominae). The genera that transmit this disease to humans are *Lutzomyia* in the New World and *Phlebotomus* in the Old World [1]. HVL is mainly affects poor people who are suffering from malnutrition, and is also a serious public health problem in many tropical and subtropical regions of the world. It is endemic in 88 countries of the world including tropics, sub-tropics and the Mediterranean basin (WHO, 1984) among which 16 are developed countries and 72 are developing countries. HVL is one of the top 10 diseases of concern to the Office International des Epizooties (OIE) and top 2 tropical parasitic diseases according to World Health Organization, is rapidly spreading, often in association with HIV infection, especially in sub-Saharan Africa and South America. Recently, it was estimated that 12 million people were infected and 350 millions were at risk of acquiring infection. HVL has emerged as an opportunistic infection in HIV-positive patients. The incidence of HIV infection has been rapidly increasing, which may lead to increasing number of Kala-azar patients. Signs and symptoms include fever, weight loss, mucosal ulcers, fatigue, anemia and substantial swelling of the liver and spleen.

Leptin is a non-glycosylated, 167 amino acids containing protein with molecular weight of 16-kDa, encoded by the obese (*ob*) gene, which is located on human chromosome 7. White adipose tissue is the major source of leptin production [2]. Leptin functions as a hormone as well as a cytokine (adipokine). As a cytokine, it attributes to various functions such as hematopoiesis, angiogenesis, and innate and adaptive immunity [2–6]. Leptin belongs to a family of class I cytokines, which are characterized by a four α-helix bundles [7].

Th1/Th2 polarization in HVL

During visceral leishmaniasis in humans the immune response is predominantly Th2 type, with absence of IFN-γ to *Leishmania* antigens [17,18] and the production of IL-10 and IL-4. IL-10 producing $CD25^-$ T cells were recently implicated in the pathogenesis of HVL in India [9]. Protective immunity in *Leishmania* infections is generally attributed to a Th1 immune response that generates IFN-γ and IL-2 producing $CD4^+$ cells. IL-12 plays an important role in the activation of Th1 cells to secret IFN-γ and IL-2 [21,22].

Leptin induces protective Th1 response

Studies on *Ob/ob* (leptin deficient) mice appeared to show an incredible role of leptin in inflammation and autoimmunity. Leptin deficient mice have reduced secretion of IL-2, IFN-γ, and IL-18 and increased production of Th2 cytokines (IL-4 and IL-10). Leptin

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A rapid method to assess the stage differentiation in *Leishmania donovani* by flow cytometry

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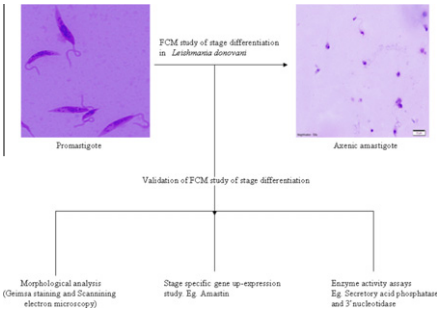
HIGHLIGHTS

- FCM is a non-flourimetric tool to study stage differentiation in *Leishmania donovani*.
- FCM was validated using different molecular and enzymatic assays.
- Partial CDS sequence (472 bp) of amastin gene was submitted in GenBank (HQ840734).
- Successful differentiation and propagation of LEM 138 were established.

GRAPHICAL ABSTRACT



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ABCA3
ATP binding cassette A3
SAcP
Secretory acid phosphatase
3′-Nucleotidase.

ABSTRACT

In this study we describe a rapid and novel method to assess the morphological stage differentiation in *Leishmania donovani* by flow cytometry (FCM). FCM is fast, accurate, and inexpensive to study the stage differentiation of promastigote into *L. donovani* axenic amastigote (LdAxAm). The non-flourimetric FCM method is easy to perform; with requirement of little expertise, and provides unambiguous results. It is an advanced tool, requires minimal time, and no fluorescent dyes. The gradual increase of differentiation and reduction in size from promastigote stage to LdAxAm leads to peak shifting from right to left on histogram. Earlier reports assessed the stage differentiation of *Leishmania* by studying the expression of stage specific markers like surface or secretory proteins and genes. For validation, conventional methods like microscopic analysis are used. These methods are quite expensive, laborious and time consuming. Non-flourimetric morphological parameters were further validated by conventional methods like optical and scanning electron microscopy. Additionally, differential expression of stage specific genes (e.g. upregulation of amastin and ATP binding cassette A3 (*ABCA3*) transporter gene transcripts) and differential activity of enzymes (down regulation of secretory acid phosphatase (SAcP) and 3′-nucleotidase enzyme activity) in LdAxAm suggest stage differentiation. Therefore, we believe that our method is an alternative tool for high reproducibility and reliability in assessment of stage differentiation.

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An *in vitro* study of apoptotic like death in *Leishmania donovani* promastigotes by withanolides

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ABSTRACT

The aim of this study was to isolate and evaluate the withanolides in inducing apoptotic like death in *Leishmania donovani in vitro*. Withanolides were fractionated and isolated from the leaves of *Withania somnifera* and LC-MS/MS analysis of two fractions namely, F5 and F6 of ethanolic extracts, obtained through column chromatography with silica gel, was performed. The antileishmanial effect of withanolides on *L. donovani* promastigotes was assessed *in vitro* using PI dye exclusion test. The effect of withanolides on promastigote morphology was determined by scanning electron microscopy. To understand their mode of action against *L. donovani*, DNA fragmentation, quantification of parasites at sub G_0/G_1 phase, determination of phosphatidylserine externalization, measurement of reactive oxygen species (ROS) and mitochondrial membrane potential (Ψ_m) were done. Results showed that LC–MS/MS analysis confirmed the presence of withanolides in isolated fractions. Treatment with withanolides resulted in morphological alterations from spindle to round shape and loss of flagella/cell integrity in promastigotes. Moreover, it induced DNA nicks, cell cycle arrest at sub G_0/G_1 phase and externalization of phosphatidylserine in dose and time dependent manner via increase in ROS and decrease in Ψ_m. Results of this study indicate that withanolides induce apoptotic like death through the production of ROS from mitochondria and disruption of Ψ_m in promastigotes of *L donovani*.

1. Introduction

Leishmaniasis is an endemic disease in 98 countries worldwide with 200 to 400 thousands new cases each year [1]. It is caused by the various species of the genus *Leishmania*. *L. donovani* is a causative agent of visceral leishmaniasis (VL) or "Kala-azar" in India. The parasite completes its life cycle in two hosts, namely sand fly and humans [2]. VL is the most severe form of leishmaniasis which is fatal, if left untreated. Approximately, world's 50% VL cases are from Indian subcontinent [3]. Till date, there has been no effective vaccine against leishmaniasis and the treatment relies exclusively on chemotherapy. Pentavalent antimonials have been the mainstay of therapy for all forms of leishmaniasis for last seven decades, however, its efficacy has declined in recent years with the result that only about one third of patients respond to it [4,5]. Efficacy of miltefosine, the drug chosen for the Elimination programme in the Indian subcontinent, has declined over the years as well [6]. Hence, there is an urgent need for the development of effective, cheaper and safer drugs to combat this disease.

Traditionally, plants have been used for the treatment of protozoan diseases [7] and plant products may play a significant role in the search of a new antileishmanial compounds. *Withania somnifera* (WS), also known as Ashwagandha is being used as an important medicinal herb for over 3000 years [8]. It has been used for various purposes ranging from an antioxidant to antibacterial [9–13]. The biological activity of WS leaves is mainly attributed to withanolides which comprises withaferin A, withanolides A-y, withanone, *etc.*, which are C_{28} steroidal lactone triterpenoids with ergosterane framework [14,15]. Mainly, much of the pharmacological activities are attributed to the two withanolides namely, withaferin A and withanolide D.

In this study, we evaluated the antileishmanial role of the withanolides isolated from the ethanolic extract of WS leaves through column chromatography with silica gel. Two fractions named as F5 and F6, at a dosage of 60 μg/ml and 15 μg/ml respectively, induce apoptotic like death in the promastigote stage of the *L. donovani in vitro*. The antileishmanial effect was found to be exerted through morphological alterations like cell shrinkage, DNA fragmentation, externalization of phosphatidylserine, induction of ROS ultimately leading to the loss of mitochondrial membrane potential. These observations clearly indicate that withanolides induce apoptotic like death in the promastigote stage of the parasite.

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Full length article

In vitro and *in vivo* evaluation of anti-leishmanial and immunomodulatory activity of Neem leaf extract in *Leishmania donovani* infection

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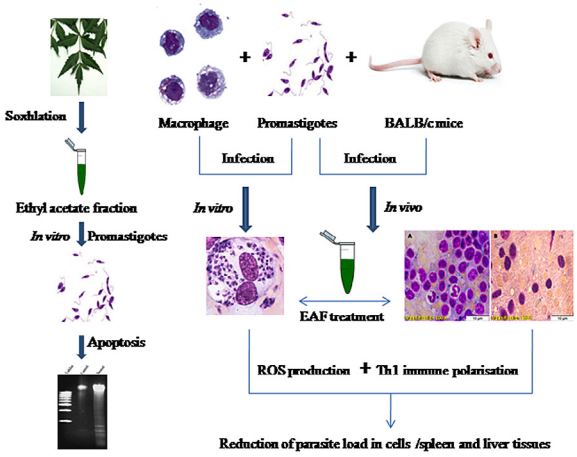
HIGHLIGHTS

- Anti-leishmanial activity of Neem leaf ethyl acetate fraction on promastigotes.
- Anti-proliferation activity and apoptosis like death in promastigotes.
- Anti-leishmanial activity on intracellular amastigotes both *in vitro* and *in vivo*.
- Th1 polarization and Th2 downregulation at m-RNA level by real-time PCR analysis.
- Increased production of reactive oxygen and nitrogen species from macrophages with extract treatment.

GRAPHICAL ABSTRACT



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ABSTRACT

The toxicity and emergence of resistance to available chemical drugs against visceral leishmaniasis is evoking to explore herbal treatment. One such attempt with the Neem is being reported here. The current study is primarily focused to evaluate the anti-leishmanial effects of Neem leaf extracts. Among which, ethyl acetate fraction (EAF) alone was found to exhibit leishmanicidal effect validated through cytotoxicity assay and estimated its IC_{50} to be 52.4 μg/ml on the promastigote stage. Propidium iodide (PI) staining of dead cells substantiated the aforementioned activity. Carboxy fluorescein–diaceate succinimidyl ester (CFSE) staining of promastigotes has affirmed its anti-proliferation activity. The characteristic features such as DNA fragmentation, reduced mitochondrial membrane potential, increased sub G_0/G_1 phase parasites and increased reactive oxygen species (ROS) production in EAF treated promastigotes indicate the apoptosis like death. In addition, the reduced parasite burden both *in vitro* (*viz.* ~45% in human monocytic leukemia cell line (THP-1) and ~50% in peripheral blood mononuclear cells) and *in vivo* (spleen and liver) provides the evidence for its anti-leishmanial activity on amastigote stage. The increase of ROS levels

Abbreviations: EAF, ethyl acetate fraction; VL, visceral leishmaniasis; THP-1, human monocytic leukemia cells; PBMCs, peripheral blood mononuclear cells; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ROS, reactive oxygen species; Ψ_m, mitochondria membrane potential; MFI, mean fluorescence intensity; LDU, Leishman–Donovan units; NO, nitric oxide.

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RESEARCH ARTICLE

Exploring the inhibitory activity of Withaferin-A against Pteridine reductase-1 of *L. donovani*

Sambamurthy Chandrasekaran[1], Jalaja Veronica[1], Ravi Kumar Gundampati[2], Shyam Sundar[3], and Radheshyam Maurya[1]

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Abstract

Withaferin A is an abundant withanolide present in *Withania somnifera* leaves and to some extent in roots. It has been known for its profound anti-cancer properties, but its role in counteracting the *Leishmania donovani* infection has to be explored. Pteridine reductase 1 (PTR1) is involved in pteridine salvage and an important enzyme for the parasite growth, which could be targeted for the development of an efficient antileishmanial drug. We employed molecular docking studies to identify the binding mode of withaferin A with PTR1 *in silico*. We further cloned, expressed, and purified PTR1 of *L. donovani* and performed the enzyme kinetics using the Michaelis–Menten equation and enzyme inhibition studies with withaferin A by plotting the Lineweaver–Burk graph, which followed an uncompetitive mode of inhibition. We also showed the inhibition of the enzyme in the crude lysate of treated parasites. Thus, our study contributes towards understanding the mode of action of withaferin A against *L. donovani* parasite.

Keywords

Autodock, *Leishmania donovani*, Pteridine reductase 1, uncompetitive inhibition, Withaferin A

History

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Introduction

According to the WHO statistics, 1.5–2 million new leishmaniasis cases are being reported annually which affects 12 million people living in 88 countries[1]. This disease is more prevalent in low-income groups residing in the endemic region, out of which 50% of the visceral leishmaniasis (VL) cases are from the Indian subcontinent[2]. Current chemotherapy for leishmaniasis includes pentavalent antimonials, amphotericin B, and more recently introduced first oral drug miltefosine. But the factors like emergence of resistance, severe side effects, high cost, and low efficacy have been hindering the usage of these drugs [3–5]. The failure of these drugs in chemotherapy necessitates for the development of alternative drugs. In this regard, our previous study showed the effective antileishmanial activity of withanolides through apoptotic like death mechanism[6] and withaferin A is an abundant compound among the withanolides in the *W. somnifera* leaves. Therefore, understanding the novel biochemical pathways in the parasite will pave path for developing selective antileishmanial drugs. In this regard, we chose PTR 1 as the target enzyme, which is exclusively present in *Leishmania* parasites and is very essential for the growth of the parasites [7,8].

Leishmania and other trypanosomatid protozoans are auxotrophs for reduced pteridines (pterins and folates), which are required for critical cellular pathways like nucleic acid and protein biosynthesis. The predominant role of PTR1 is to salvage oxidized pterins rather than to reduce folates and it is the only enzyme that has been reported to reduce biopterin for the *in vivo* growth of *Leishmania* [8–11]. Thus, pterin compounds like biopterin or folate are acquired from the host and active tetrahydro-species are generated by successive reductions of pterin compounds that are carried by two bifunctional enzymes namely dihydrofolate reductase-thymidylate synthase (DHFR-TS) and pteridine reductase (PTR1). The former reduces folate and 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF)[7] and the latter catalyzes NADPH-dependent reversible reduction of oxidized pterins to dihydrobiopterin (DHB) as well as of tetra-hydrobiopterin (THB) and folates to DHF and THF[7]. Although it catalyzes the same reaction as that of DHFR, the failure of anti-folate strategies lies in the PTR1 resistance to DHFR inhibitors like methotrexate. The intriguing feature of PTR1 makes it a suitable drug target for the development of antileishmanial agents [7,12]. In this regard, PTR1 presents an attractive drug target for the development of antileishmanial agents.

Withania somnifera is also known as Ashwagandha or Indian ginseng in the Ayurvedic medical systems. It has been used as an indigenous herb for more than 3000 years[13]. The pharmacological activities of this plant have been attributed to its secondary metabolites called withanolides[14]. Withanolides are C_{28}-steroidal lactones with the intact or rearranged ergostane framework. Withaferin A is one of the most predominant compounds among the withanolides. Previous studies have reported various biological activities of the crude root[15] and leaf extracts[16–18] of

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Full length article

Leptin induces the phagocytosis and protective immune response in *Leishmania donovani* infected THP-1 cell line and human PBMCs

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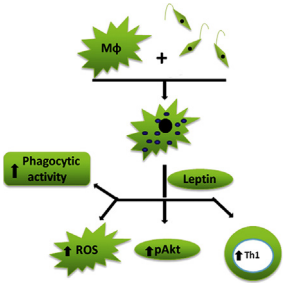
HIGHLIGHTS

- Leptin induces *L. donovani* parasite uptake by macrophages of both THP-1 and PBMCs.
- Leptin upregulates the reactive oxygen species generation in infected THP-1.
- Leptin enhances the phosphorylation of Akt and Erk1/2 signaling in infected THP-1.
- Leptin enhances the proinflammatory cytokine response against *L. donovani* infection in both THP-1 and PBMCs.

GRAPHICAL ABSTRACT



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ABSTRACT

Visceral leishmaniasis (VL) is an infectious disease responsible for several deaths in malnourished children due to impaired cell-mediated immunity, which is accompanied by low circulating leptin levels. The cytokine function of leptin is implicated for several immune regulation activities such as hematopoiesis, angiogenesis, innate and adaptive immunity. Its deficiency associated with polarization of Th2 response, which coincides with VL pathogenesis. To determine the cytokine role of leptin in case of experimental VL, we tested the leptin associated Th1/Th2 type cytokine profile at mRNA level from *Leishmania donovani* infected human monocytic leukemia cell line (THP-1) and peripheral blood mononuclear cells (PBMCs). We also tested the effect of leptin on macrophages activation (viz. studying the phosphorylation of signaling moieties), phagocytic activity and intracellular reactive oxygen species (ROS) production during infection. We observed that leptin induced Th1 specific response by upregulation of IL-1α, IL-1β, IL-8 and TNF-α in THP-1 and IFN-γ, IL-12 and IL-2 in PBMCs. We also observed the downregulation of Th2 type cytokine i.e. IL-10 in THP-1 and unaltered expression of cytokines i.e. TGF-β, IL-10 and IL-4 in PBMCs. In addition, leptin stimulates the macrophages by inducing phosphorylation of Erk1/2 and Akt which are usually dephosphorylated in *L. donovani* infection. In concordance, leptin also induces the macrophage phagocytic activity by enhancing the intracellular ROS generation which helps in phagolysosome formation and oxidative killing of the parasite. In compilation, leptin is able to

Abbreviations: VL, visceral leishmaniasis; THP-1, human monocytic leukemia cell line; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; Erk1/2, extracellular-regulated kinase 1/2; Akt, protein kinase B; IL, interleukin; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; CD, cluster differentiation factor; MFI, mean fluorescence intensity; H_2DCFDA, 2, 7-dichlorodihydrofluoresceindiacetate; RIPA, radioimmunoprecipitation assay.

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understanding the molecular mechanisms implicated in resistance of

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4	Kato, Hirotomo, Eduardo A. Gomez, Abraham G. CÃ¡ceres, Hiroshi Uezato, Tatsuyuki Mimori, and Yoshihisa Hashiguchi. "Molecular Epidemiology for Vector Research on Leishmaniasis", International Journal of Environmental Research and Public Health, 2010. Publication	1%