Studies on Drug Sensitivity in *Leishmania donovani* Field Isolates and Differential Gene Expression Analysis in Miltefosine Resistant Parasites

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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Under the Supervision of

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

2012

BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE

PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled '**Studies on Drug Sensitivity in *Leishmania donovani* Field Isolates and Differential Gene Expression Analysis in Miltefosine Resistant Parasites'** and submitted by Arpita Kulshrestha ID No. 2007PHXF436 for award of Ph.D. Degree of the Institute, embodies original work done by her under my supervision.

Signature in full of the Supervisor _____

Name in capital block letters _____

Designation _____

Date _____

Acknowledgement

Acknowledgment is the only means to express the deep sense of gratitude towards the support and inspiration of all those people who remained associated with me directly or indirectly all through the tenure of my thesis work, and paved the path towards successful accomplishment of arduous task like this. Though the written words have a general tendency to mislead the feeling of gratitude to bland formality, it is the only means to make the feeling of "Thanks" permanent.

The studies in this thesis were carried out at Laboratory of Molecular Parasitology, National Institute of Pathology (ICMR), New Delhi, India. The financial support from ***Indian Council of Medical Research*** *(ICMR) and* ***Council of Scientific and Industrial Research*** *(CSIR), India is gratefully acknowledged.*

Very few personalities have a rare combination of a good professional as well a good human being in this world and I am profoundly blessed in finding one. I wholeheartedly express sincere thanks to that personality as my Ph.D. guide and mentor ***Dr. Poonam Salotra****, Deputy Director and Scientist E, National Institute of Pathology (ICMR) for her intellectual and dynamic guidance, exceptional ideas, invaluable insights, tireless optimism, incomparable support, enthusiasm and encouragement throughout my doctoral studies. My gratitude towards her is beyond the limit of words. Her capacity to combine critique with an immediate empathy and commitment towards workers and others engaged in struggle will always inspire me. Besides the illuminating scientific guidance, her all time support, encouragement have been the main driving forces behind the successful completion of my research work. It was a matter of fortune for me to accomplish my research work under her guidance.* ***"Thanks Ma'am"*** *for your dynamic guidance and encouragement.*

I express my sincere thanks to ***Dr. Sunita Saxena****, Director, National Institute of Pathology (ICMR), for providing me the opportunity to avail the research facilities of the institute and for her constant encouragement.*

I am immensely thankful to ***Prof. B. N. Jain****, Vice-Chancellor, BITS, Pilani for providing me this opportunity to pursue the off-campus PhD of the Institute. I express my gratitude to* ***Prof. A. K. Das****, Dean, Research and Consultancy Division (RCD), BITS, Pilani for your constant official support, encouragement and making the organization of my research work through the past few years easy.*

I am thankful to you ***Dr. Dinesh Kumar, Dr Sharad Shrivastava and Dr. Monica Sharma,*** *nucleus members of RCD, BITS, Pilani, without your cooperation and guidance it would not have been possible for me to pursue such goal oriented research during each of the past few semesters.*

I sincerely express my thanks to ***Dr. V. Ramesh****, Professor and Head, Department of Dermatology, and* ***Dr. N. S. Negi****, Senior Specialist, Department of Medicine, Safdarjung Hospital, New Delhi for wholehearted support and providing me the clinical samples, which were the building blocks of my research experiments.*

I wish to recall all the guidance and help I received from ***Professor Indira Nath, Dr. Aruna Mittal, Dr. Sujala Kapoor, Dr. A. K. Jain, Dr. Sangeeta Rastogi, Dr. Saurabh Verma, Dr. L. K. Yerneni, Dr. Usha Aggarwal, Dr. A. K. Mishra, Dr. Avninder Singh*** *and* ***Dr. L.C. Singh****.*

I am indebted with sincere thanks to ***Dr. Hira L Nakhashi*** *and* ***Dr. Robert C Duncan****, CBER, FDA, USA and* ***Dr A. Selvapandiyan*** *for the ideas and discussions at several crucial stages of my research work.*

I could always rely on ***Mrs Saratha,*** *Administrative Officer, National Institute of Pathology (ICMR), for her administrative help in countless instances and express my deep felt thanks to her.*

Sincere note of thanks to ***Mr Anish, Mr Ramchandra*** *and* ***Kamlesh ji****, their ready help and cooperation during every need made my endeavor much more convenient in the hour of need.*

I am thankful from the bottom of my heart to all my seniors, friends and juniors whose company has made this long and arduous journey interesting and enjoyable. I heartily acknowledge my seniors ***Dr. Nasim Akhtar Ansari, Dr Ruchi Singh, Dr BVD Raju, Dr Srividya Gurumurthy, Dr Rajesh Kumar, Dr Paresh Sharma*** *and* ***Dr Dhiraj Kumar****, for their support and with whom I spent a fruitful time. My special thanks to* ***Sandeep*** *who was always there as a friend and guide, providing an easy atmosphere to work with. I will always be thankful to him for encouraging me to do the things in a systematic way. I would also like to thank* ***Anand Raj,*** *who was there for me during the hour of need and for his selfless support.*

I am grateful to my lab mates ***Vasundhra, Himanshu, Vanila, Avishek*** *and* ***Deepak****, not only for their ready help and cooperation but also for providing a loving and friendly atmosphere in the lab, which made the work enjoyable. The enthusiasm and support provided by my friends as well as seniors* ***Dr. Hem Chand Jha, Dr. Rajnish Jha, Dr. Rishein Gupta, Dr Harsh Vardhan, Pragya, Abha, Apurb, Dhirendra, Regina, Mishi, Rakshan, Pradeep, Vaani and Ira*** *is gratefully acknowledged.*

Some people somewhere somehow walk into your life, whose contribution in your day-to-day life cannot be acknowledged, but can only be felt from heart; ***Gajendra*** *is that special one in my life. He was a source of constant moral support during my most difficult days. Not only on personal front but also on professional front I am indebted towards his help and support. He was always there for me as a companion to work along and now a companion for life. I convey my heartiest regards to* ***Daddy*** *and* ***Mummy ji*** *for providing me constant encouragement and support.*

At this moment, my heart goes towards my ***Grandparents*** *and* ***Parents*** *for their blessings, who waited all these almost endless years of my study to see me reaching at this stage. My gratitude towards* ***Papa*** *and* ***Maa*** *knows no bounds. I am what, I am because of them; you are the wind beneath my wings. I am forever grateful to them, whose foresight and values paved the way for a privileged education, and they gently offered counsel and unconditional support at each turn of the road. Their constant patience, prayers and blessings motivated and inspired me to do the things in a perfect manner. It is the strength of struggle and dreams in the eyes of my parents that always motivated me to move ahead. I am thankful to my God for blessing me with the best parents in the world, who always stood by me in all my decisions with their best suggestions and utmost care. Love and affection of my brother,* ***Apar*** *was always a source of courage in my hard times. I wish to express my deep sense of gratitude to my teachers for their proper guidance, encouragement and blessings. Last but not the least I am grateful to my patients without whose support, the study would not have been possible.*

Above all, I am thankful to ***Almighty God*** *for not letting me down both morally and physically during the hard days of my struggle.*

Great thanks to all of you once again.

April 2012 ***Arpita Kulshrestha***

Contents

Abbreviations

AmB	AmphotericinB
A_{280}	Absorbance at 280 nm
A_{540}	Absorbance at 540 nm
A_{600}	Absorbance at 600 nm.
Am	Amastigote
Amp	Ampicillin
ATP syn	ATP synthase
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BMA	Bone marrow aspirates
BSA	Bovine Serum Albumin
^{o}C	Degree Celsius
cDNA	Complementary DNA
CL	Cutaneous Leishmaniasis
CytB5	CytochromeB5
CytB5 red	CytochromeB5 reductase
dATP	Deoxyadenosine tri phosphate
dCTP	Deoxycytidine tri phosphate
dGTP	Deoxyguanosine tri phosphate
ddH_2O	Double Distilled water
DMSO	Dimethylsulfoxide
ddNTP	Di-Deoxyribose nucleotide tri phosphate
DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine tri phosphate
dUTP	Deoxyuridine tri phosphate
dpm	Disintegrations per minute

EDTA	Ethylene diamine tetra-acetic acid
ED_{50}	Effective Dose that kills 50% of microbial population
ED_{90}	Effective Dose that kills 90% of microbial population
ER	Endoplasmic reticulum
FCS/FBS	Fetal Calf/Bovine Serum
FFA	Free Fatty acid
g	Gravitational force
Gms	Grams
GSH-	Glutathione
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
HEPES	N-2-hydroxyethylpiperazine-N-2 ethane sulfonic acid
hr/hrs	Hour/hours
HiFCS	Heat inactivated Fetal Calf Serum
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HASPA	Hydrophilic acylated surface protein A
HR	High Resistance zone to Antimony
HSP	Heat shock protein
IFA	Immunofluorescence assay
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-b-D-thio-galactosidase
KA	Kala-azar
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
Ld	*Leishmania donovani*
Lm	*Leishmania major*
L-NMMA	N-methyl-L-arginine monoacetate
LPL	Lipase Precursor Like protein
LPS	Lipopolysaccharide
LR	Low Resistance Zone to Antimony

M	Molarity
MAP kinase	Mitogen activated protein kinase
MIL	Miltefosine
mCi	Millicurie
mg/ ml	Milligram/ Milliliter
min/mins	Minute/Minutes
Mit IM	Mitochondrial inner membrane
Mit OM	Mitochondrial outer membrane
MOPS	3-[N-Morpholino] propanesulfonic acid
NDDH	NAD/FAD dependent dehydrogenase:
$NaHCO_3$	Sodium bicarbonate
N	Normality
ND	Not Determined
ng	Nanogram
NO	Nitric oxide
NOS	Nitric Oxide Synthase
NR	Non responder
O/N	Over night
OD	Optical Density
ORF	Open Reading Frame
PAGE	Poly Acrylamide Gel Electrophoresis
PBS/ PBS-T	Phosphate Buffered Saline/ PBS with Tween 20
PCR	Polymerase chain reaction
PG Mut	Phospho glycerate mutase
PK	Protein kinsae
PKDL	Post Kala-azar Dermal Leishmaniasis
PMM	Paromomycin
PMSF	Phenyl Methyl sulfonyl fluoride
PPG	Proteophosphoglycan
Pro	Promastigotes
PSA	Parasite surface antigen

r	Correlation coefficient
rpm	Revolution per minute
RNase	Ribonuclease
RPMI	Roswell Park memorial Institute
RT-PCR	Reverse transcription- PCR
qRT-PCR	Quantitative real-time PCR
SAG	Sodium Antimony Gluconate
SSG	Sodium Stibo Gluconate
Sec/Secs	Second/Seconds
SD	Standard Deviation
SEM	Standard Error Mean
SJH	Safdarjung Hospital
SHERP	Small Hydrophilic Endoplasmic Reticulum Associated Protein
SIT	Sitamaquine
SSC	Sodium chloride sodium citrate
TAE	Tris acetate EDTA
TAG	Tri Acyl glycerol
TEMED	N,N,N',N' tetramethyl ethylene diamine
Tob	Topogenesis of mitochondrial outer membrane β-barrel proteins
TOM	Translocators of Outer Membrane
Tris	Tris (hydroxymethyl) amino acid
[T(SH)2]-	Trypanothione
(TrS)-	Trypanothione synthetase
TP(ox)-	Tryparedoxin peroxidase,
U	Unit
UV	Ultra Violet
VL	Visceral Leishmaniasis
WT	Wild type
X g	Times gravity (centrifugal force)

X-gal	5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside
b-ME	Beta mercaptoethanol
mg/ml	Microgram/ Microliter
mM/mM	Micromolar/ Millimolar
%	Percentage
~	Approximately

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Introduction

Leishmaniasis is a group of diseases with wide epidemiological and clinical diversity caused by protozoan parasite, *Leishmania*. The disease is endemic in large areas of the tropics, subtropics and the Mediterranean basin. Leishmaniasis is characterized by both diversity and complexity (Herwaldt, 1999): it is caused by more than 20 *Leishmania* species and is transmitted to humans by more than 30 different species of Phlebotomine sandflies (Pearson & Sousa, 1996).

The two major clinical forms of Leishmaniasis, cutaneous and visceral, are the result of infection by different species of the parasite. However, in addition to the infecting species, the clinical outcome of Leishmaniasis also depends on the immune response of the host (Rivas et al., 2004). Environmental risk factors such as massive displacement of populations, urbanization, deforestation, new irrigation plans and individual risk factors such as HIV, malnutrition and genetic susceptibility make Leishmaniasis an important public health problem (Desjeux, 2004).

Visceral Leishmaniasis (VL) is a lethal, disseminated form of this infection caused by *L donovani/ L infantum* and stands second only to malaria as a parasitic cause of death. Prevalent in 70 countries, 90% of the VL cases occur in India, Bangladesh, Nepal, Brazil and Sudan (Desjeux, 2004). India alone shares almost 50 % of the world`s global VL burden. In India, states of Bihar, Uttar Pradesh and West Bengal are highly endemic foci of VL or kala azar (KA) where periodic epidemics are common (Adhya et al., 2002). The official estimate of 430,000 VL cases in Bihar state over the past 11 years may represent only a fraction of the real numbers. The actual number is believed to be at

least 5 times as great (WHO, 2004). This has compelled the governments of India, Nepal and Bangladesh to launch regional VL elimination programme (WHO, 2004; Chappuis et al., 2007). In the absence of vaccines and limited impact of vector control, chemotherapy is the key strategy in VL control and cure (Olliaro et al., 2005; Jha, 2006; Sundar & Chatterjee, 2006). VL treatment relied in the past on the chemotherapy with antimonials (Sodium antimony gluconate; SAG). However, in parts of India, specifically Bihar, resistance to SAG has emerged, rendering it useless in more than 60% VL patients (Croft et al., 2006). In consequence, treatment costs have risen because of increased doses, prolonged hospitalization, and need for retreatment. Hence, alternative drug treatments in VL endemic areas are badly needed.

New treatments for VL have been introduced and others are on clinical trials. Amphotericin B (AmB), a polyene macrolide antifungal agent has shown to be highly effective in the treatment of VL. However, it has the disadvantage of being administered as slow intra venous infusions and causes potentially serious renal toxicity (Sundar & Rai, 2005). Most promising treatments like liposomal AmphotericinB (L AmB) that have been found to be effective even at low doses in India (Sundar et al., 2002a) and Brazil (Berman et al, 1998), are not affordable for most of the patients in endemic countries. Moreover, unresponsive cases for L AmB have also shown up in Sudan (Mueller et al., 2007). Miltefosine (MIL), an alkyl phospholipid, is the first oral drug for VL in India, registered in 2002. It has proved to be highly effective in treatment of VL in India both in adults (Sundar et al., 1998; Sundar, 2001) and in children (Bhattacharya et al., 2004). Aminoglycoside paromomycin is recently registered as antileishmanial drug in India and has been found to be effective in comparison to SAG in phase trials (Sundar et al., 2007;

Sinha et al., 2011). Sitamaquine, an 8 amino-quinoline, is still under phase II trials (Jha et al., 2005; Wasunna et al., 2006). Care needs to be taken that resistance does not develop to these drugs. Hence, drug susceptibility monitoring is crucial to prevent the emergence of resistance to the new drugs for effective control of Leishmaniasis.

In India, Bangladesh and Nepal, monotherapy with MIL has been proposed as first line treatment even in areas where SAG is effective (WHO, 2004). In India, where anthroponotic transmission of VL occurs, widespread use of MIL monotherapy might lead to the rapid emergence of clinical resistance (Sundar et al., 1998; Bryceson, 2001). MIL has a long terminal half life (150-200 hrs), increasing the risk of development of resistant parasites in the field that could lead to rapid transmission of MIL-resistant population and put the lifespan of this important drug at risk as exemplified by SAG resistance in India. Moreover, resistance to this drug was shown to be readily induced *in vitro* (Perez –Victoria et al., 2006). In this situation; it becomes very crucial to understand the exact mechanism of action of MIL and the resistance mechanism that might arise in future.

One of the goals of the present study is to evaluate inherent susceptibility of the presently prevailing parasite population in India towards currently available (AmphotericinB, Miltefosine) and new drugs (Paromomycin, Sitamaquine). This will help to determine any correlation between susceptibility towards SAG and these antileishmanial drugs and to assess any cross resistance mechanism to these drugs in SAG resistant isolates. Apart from these antileishmanial drugs, towards development of safe, effective and affordable chemotherapeutic options, natural products like extracts from marine organisms will also be evaluated for their potential antileishmanial activity.

The highly effective oral antileishmanial drug MIL is at a high risk of emergence of resistance. There is hence an urgent requirement to define resistance mechanism of this drug. Drug resistance is a complex phenomenon in the *Leishmania* parasite and several metabolic pathways and membrane transporters are implicated in the resistant phenotype. Microarray technique is a high throughput approach for large-scale gene expression profiling and enables the investigation of fundamental processes and the molecular basis of the disease on genomic scale. The study therefore aims to define the genetic determinants of MIL resistance in *Leishmania* using genomic microarray technique. The interacting genes and pathways that will be identified through this study are most likely to provide an understanding of the resistance mechanism of the parasite, identify potential biomarkers of MIL resistance and thereby improve the therapeutic index of this valuable class of antileishmanial drug.

Review of Literature

1.1. Historical background of Leishmaniasis

Leishmaniasis constitutes a group of vector borne diseases, caused by more than 20 species of the protozoan genus *Leishmania*, and ranging from localized skin ulcers to lethal systemic disease. According to ranking, it is the second most prevalent parasitic disease after malaria. Leishmaniasis has been considered a tropical affliction that constitutes one of the six entities on the list of most important diseases of World Health Organization/Tropical Disease Research (WHO, 2007; Desjeux, 2004). It is classified as one of the "most neglected diseases" based on the limited resources invested in diagnosis, treatment and control, and its strong association with poverty.

Representations of skin lesions and facial deformities have been found on pre Inca pottery from Peru and Ecuador dating back to the first century AD. There are evidences that some forms of Leishmaniasis prevailed as early as this period (http://www.who.int/en). There are detailed descriptions of oriental sore by Arab physicians including Avicenna in the 10th century, who described it as Balkh sore from northern Afghanistan, and there are later records from various places in the Middle East including Baghdad and Jericho; many of the conditions were given local names by which they are still known. In the Old World, Indian physicians applied the Sanskrit term kala azar (KA) (meaning ‘black fever’) to an ancient disease that was later defined as visceral leishmaniasis (VL). KA was first noticed in Jessore in India (presently in Bangladesh) in 1824, when patients suffering from fever that was thought to be due to malaria failed to respond to quinine. By 1862, the disease had spread to Burdwan (West Bengal), where it reached epidemic proportions. In 1901, William Leishman identified certain organisms in

smears taken from the spleen of a patient who had died from ‘dum-dum fever’ (Leishman, 1901). Initially, these organisms were considered to be Trypanosomes, but in 1903, Captain Donovan described them as being new. The link between these organisms and KA was eventually discovered by Major Ross, who named them *Leishmania donovani* (Ross, 1903). The search for a vector was a long one, and it was not until 1921 that the experimental proof of transmission to humans by sandflies belonging to the genus *Phlebotomus* was demonstrated. It was later proved that the *Leishmania* parasite could be transmitted by *Phlebotomus* sandflies (Cox et al., 2002; Swaminath et al., 2006).

There are more than 21 morphologically indistinguishable species of *Leishmania* that infect humans. Conventionally, they are classified and named mainly according to their geographical distribution and clinical characteristics of the disease they afflict (Roberts et al., 1996; Herwaldt, 1999).

1.2. Risk factors and definition of the problem

The number of cases of Leishmaniasis is increasing, mainly because of manmade environmental changes that increase human exposure to the sandfly vector (Desjeux, 2004). Leishmaniasis is spreading in several areas of the world as a result of epidemiological changes which sharply increase the overlapping of acquired immunodeficiency syndrome (AIDS) and VL. Continuing widespread migration from rural to urban areas, and continuing fast urbanization worldwide are among the primary causes for increased exposure to the sandfly (Desjeux, 2004). Climate change and other environmental changes have the potential to expand the geographic range of the vectors and Leishmaniasis transmission in future (http://www.cdc.gov/parasites/

leishmaniasis/epi.html). Increasing risk factors are making Leishmaniasis a growing public health concern for many countries around the world.

1.3. Types of Leishmaniasis

Leishmaniasis causes considerable morbidity and mortality. Parasites of the *Leishmania* genus are remarkably diverse biologically, clinically, and epidemiologically, and present enormous differences in disease tropism. The disease has traditionally been classified in three major forms on the basis of clinical symptoms (Handman, 2001). The most deadly form is visceral leishmaniasis (VL), which if left untreated, leads to full blown disease and eventually, to death. A number of other species of *Leishmania* cause cutaneous (CL) and mucocutaneous (MCL) leishmaniasis, which, although not fatal, are still responsible for considerable morbidity of a vast number of people in endemic foci (Peters et al., 1995; Handman, 2001; WHO, 2008).

1.3.1. Cutaneous Leishmaniasis (CL)

This is the most common form of Leishmaniasis, also known as 'Oriental sore' which first appears as a persistent insect bite. Simple skin lesions appear at the site of sandfly bite (Fig 1.1) which self heal within few months but leaves scars.

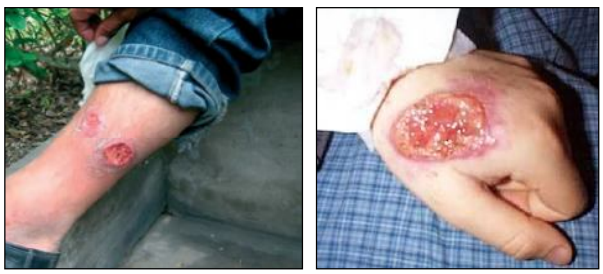


Figure 1.1: Clinical signs of Cutaneous Leishmaniasis. Patients with skin ulcers due to CL. (Reproduced from Chappuis et al, 2007, Nature Rev Microbiol, 5:S7-15 and http://www3.baylor.edu /Charles_Kemp/hand.jpg).

The incubation period can last from few days to months. Gradually, the lesion enlarges, remaining red, but without noticeable heat or pain. Resolution of the lesion involves immigration of leucocytes, which isolate the infected area leading to necrosis of the infected tissues, and formation of a healing granuloma in the floor of the lesion. It has been estimated that 90% of CL cases occur in 7 countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria.

1.3.2. Diffuse Cutaneous Leishmaniasis (DCL)

This is a chronic, progressive, polyparasitic variant that develops in context of leishmanial specific anergy and is manifested by disseminated non ulcerative skin lesions, which can resemble lesions of lepromatous leprosy (Fig 1.2). DCL is restricted to Venezuela and Dominican Republic in the western hemisphere, and to Ethiopia and Kenya in Africa. Its main causative organisms are *L. aethiopica* (old world) and *L mexicana* species complex (new world).

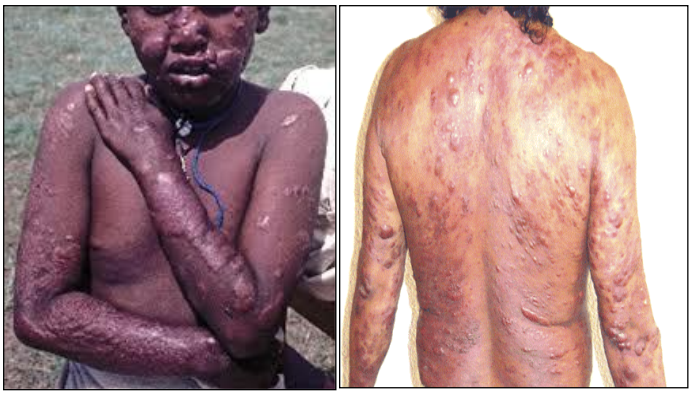


Figure 1.2: Patients with clinical symptoms of Diffuse Cutaneous Leishmaniasis. (Reproduced from otm.oxfordmedicine.com and Calvopina et al., 2006; Am J Trop Med Hyg. 75:1074-7).

1.3.3. Mucocutaneous Leishmaniasis (MCL)

This form of disease, also known as ‘‘espundia’’, causes extensive destruction of naso-oral and pharyngeal cavities with hideous disfiguring lesions, mutilation of the face (Fig 1.3) and great suffering for life. MCL is occasionally reported from Sudan and other Old World foci and is caused by *L. guyanensis, L. mexicana, L. amazonensis* and *L. panamensis*. Classical MCL is, however, restricted to *L. braziliensis* infections in which, following the apparently complete resolution of the initial oriental sore, sometimes many years later, metastatic lesions appear on the buccal or nasal mucosa.

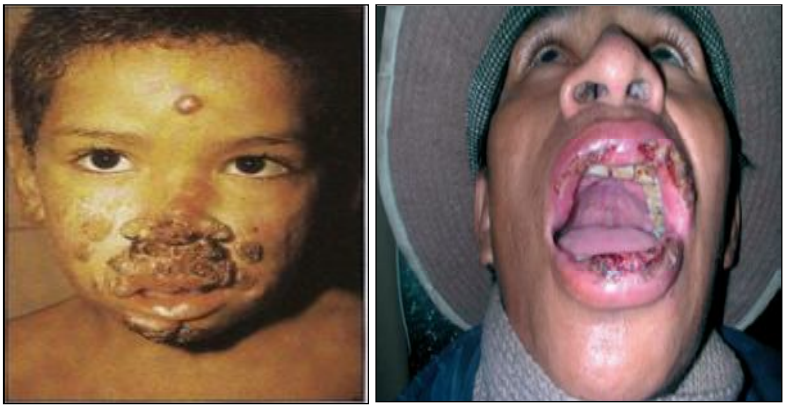


Figure 1.3: Mucocutaneous Leishmaniasis patients with perforated nasal septum and mucosal tissue destruction. (Source: Prof. Dr. Wolfgang Bommer, University Hospital Goettingen/Germany and Chappuis et al., 2007, Nature Rev Microbiol, 5:S7-15).

The reservoir hosts include rodents, opossums, anteaters, sloths and dogs etc. Human infection occurs when human invade the forest habitats. MCL is endemic in Mexico and Central and South America.

1.3.4. Visceral Leishmaniasis (VL)

VL is the most dreaded and devastating amongst the various forms of Leishmaniasis. VL is also known as kala azar, black sickness, black fever, Burdwan fever, dum-dum fever etc. It is the most severe form of disease and is usually fatal if left untreated. The parasite is responsible for a spectrum of clinical syndromes, which can, in most extreme cases, move from an asymptomatic infection to a fatal form of VL.

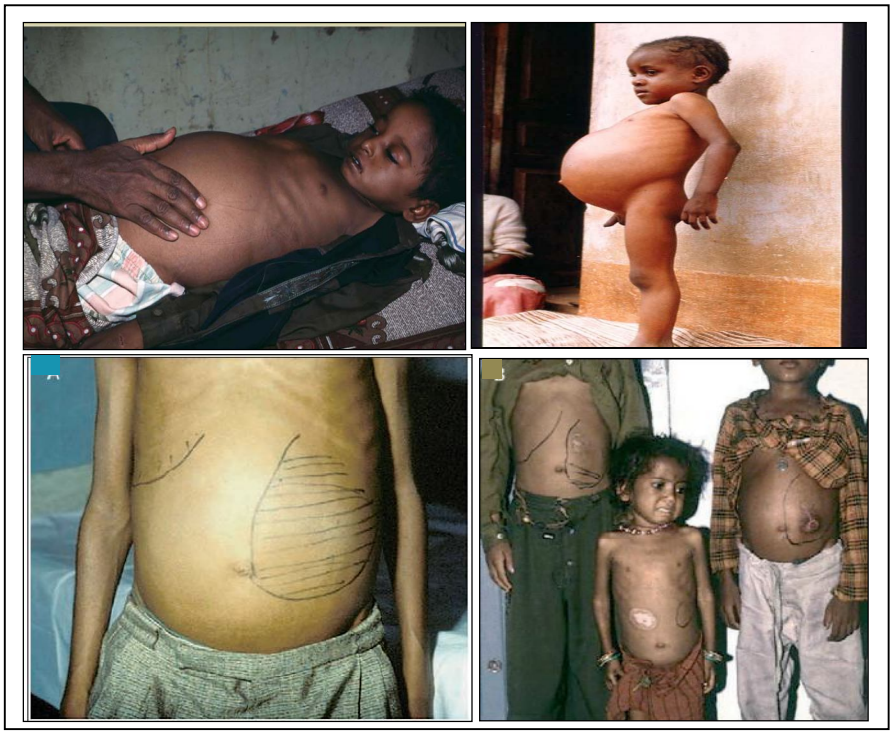


Figure 1.4: Clinical symptoms of VL. Hepato splenomegaly and wasting are the main features (Reproduced from Murray et al., 2005; Lancet 366: 1561–77 and TDR report, February 2004).

VL is characterized by fever, severe cachexia, hepatosplenomegaly (splenomegaly usually predominates) (Fig. 1.4), pancytopenia (anaemia, thrombocytopenia, and leucopenia, with neutropenia, marked eosinopenia, and a relative lymphocytosis and

monocytosis), and hypergammaglobulinaemia (mainly IgG from polyclonal B cell activation) with hypoalbuminaemia (Herwaldt, 1999). The parasite invades and multiplies within macrophages (free mononuclear phagocytic cells) and affects the reticuloendothelial system including spleen, liver, bone marrow, and lymphoid tissue (Aggarwal et al., 1999; Boelaert et al., 2000). VL encompasses a broad range of manifestations of infection which remains asymptomatic or subclinical in many cases, or can follow an acute, subacute, or chronic course. Active VL may also represent relapse (recurrence 6-12 months after apparently successful treatment) or late reactivation (recrudescence) of subclinical or previously treated infection. Risk factors for development of clinical disease include malnutrition, immunosuppressive drugs and especially, HIV co-infection.

VL results from systemic infection with parasites of *L. donovani* complex with *L. infantum* occurring in Europe, North Africa, South and Central America, (Alvar et al., 2004), *L. donovani*, found throughout East Africa, India, and parts of the Middle East and *L.chagasi* in Latin America. Evidences have accumulated that *L. infantum* and *L. chagasi* are the same species (Maurício et al., 2000). The disease burden associated with VL, measured in disability adjusted life years (DALYs), was estimated to be 1,980,000 [1,067,000 for male and 744,000 for female] (Guerin et al., 2002). VL is emerging as an important opportunistic infection among people with HIV-1 infection (Alvar et al., 1997; Desjeux & Alvar, 2003). In fact, the parasite may be a cofactor in the pathogenesis of HIV infection (Bernier et al., 1998; Thakur, 2003).

1.3.5 Post Kala azar Dermal Leishmaniasis (PKDL)

PKDL is an unusual dermatosis, usually occurring as a sequel to VL, which produces non ulcerative cutaneous lesions in the form of hypopigmented macules, erythema, and nodules (Fig.1.5). PKDL develops in about 5-15% of VL cases after months or several years of remission from infection in India and within weeks or months in 50-60% cured VL cases in Sudan (Rees et al., 1984; Salotra & Singh, 2006). Elimination of VL therefore requires accurate detection and treatment of PKDL, necessarily because of its capacity to serve as a reservoir for the causative parasite, *L. donovani* in anthroponotic foci of VL transmission such as India.

(A) (B)

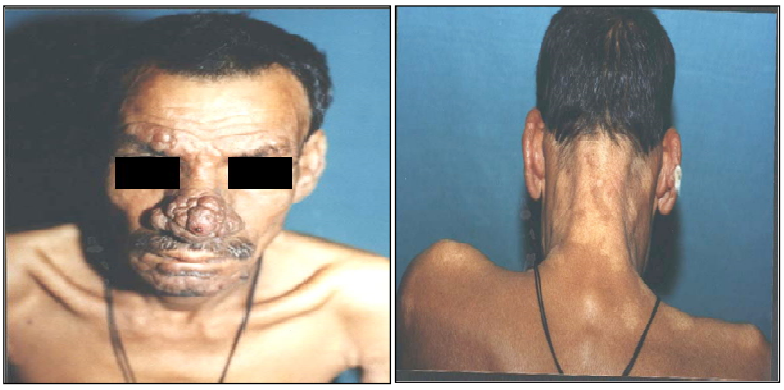


Figure 1.5: PKDL patients with (A) papular-nodular lesion (B) hypopigmented macular lesion. (Reproduced from Salotra & Singh, 2006, Indian J Med Res 123: 295).

1.4. National status of Visceral Leishmaniasis (Kala azar)

In India, a country with a high VL burden, 88% of the patients have a daily income of less than 2 USD and a poor economic level (Murray et al., 2005). The incidence of KA in India is among the highest in the world (Desjeux, 2004). In India, about 100,000 cases of VL are estimated to occur annually (WHO/TDR News, 2007) (Fig 1.6. B, C). Each year Bihar alone contributes 80-90 % of the KA cases (Zijlstra et al., 1995; Das et al., 2010). Other constraints are the positioning in health system, implementation and standardization of treatment guidelines, natural calamities like regular floods in many KA endemic districts, PKDL cases and its treatment, emerging foci of drug resistance, inadequate information on vector bionomics, asymptomatic carriers and procurement difficulties.

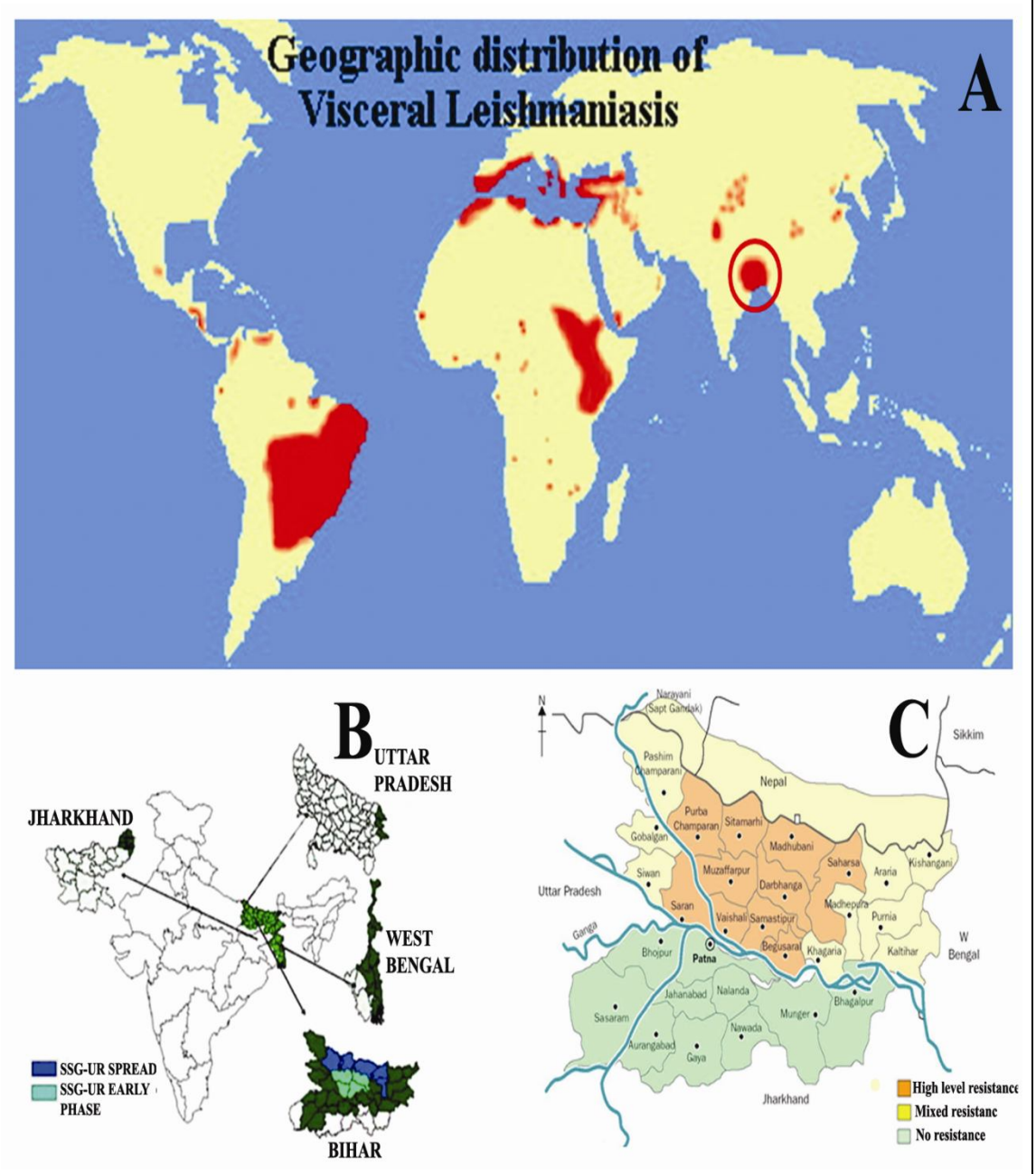


Figure 1.6: (A) Worldwide distribution of Visceral Leishmaniasis. (source: http://www.vet.uga.edu/vpp/archives/NSEP/Brazil2002/leishmania/Eng/Leish04.html)

(**B) VL affected states of India** (source: http://nvbdcp.gov.in/kala-azar.html and **(C) VL affected districts of Bihar** (Reproduced from Guerin et al., 2002; Lancet Infect Dis. 2:494-501).

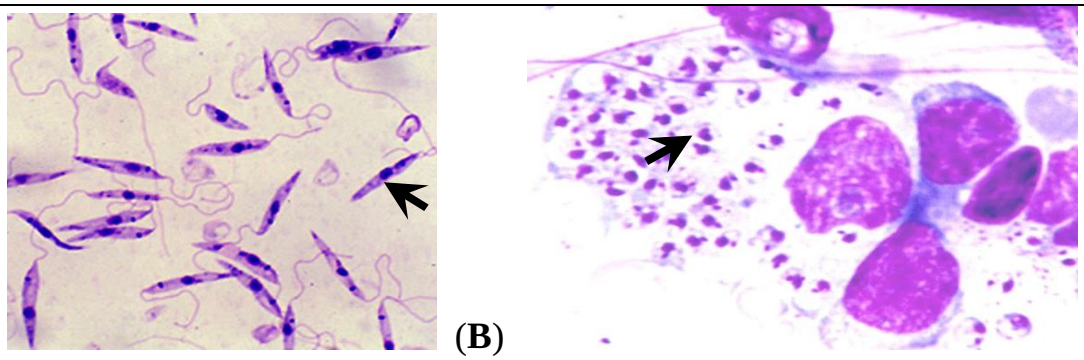
1.5. Morphology and Life cycle of *Leishmania donovani*

Indian VL is anthroponotic and is transmitted chiefly through the bites of the female sandfly, *P. argentipes*. *Leishmania* exists in two forms (i) promastigotes: these are extracellular, elongated, flagellated, motile and range in size from 2 µm ´ 2-20 mm (Fig. 1.7.1 A). This form exists in sandfly and in *in vitro* cultures (ii) amastigotes: these are intracellular, aflagellated, round to oval, non motile and range in size from 2-5 mm (Fig. 1.7.1 B). This form resides and multiplies within the phagolysosomes of macrophages of reticuloendothelial system of the vertebrate host (Handman, 2001). Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow. CL and MCL have life cycle exactly similar as that of *L. donovani* except that the amastigote form resides in the large mononuclear cells of the skin (Fig. 1.7.2).

1.5.1 ***Leishmania life cycle in the sandfly:*** After ingestion of a blood meal from an infected host, the parasites initially reside within the peritrophic membrane inside the sandfly's mid gut. Amastigotes are then released from the macrophages of the blood meal and differentiate into the promastigote stage. They now synthesize an increasingly dense coat of a glycocalyx, which is composed of a variety of glycoconjugates that are bound to the surface of the parasite by a glycophosphatidylinositol (GPI) anchor (McConville & Ralton, 1997). Underneath the glycocalyx, a dense layer of low molecular weight glycoinositolphospholipids (GIPLs) is found, which is thought to have barrier functions. Among the glycoconjugates, a lipophosphoglycan (LPG), which contains a repeating polymer of disaccharide phosphate repeating units, is the most abundant (Beverley &

Turco, 1998). After about 2 days, the promastigotes attach to the mid gut wall through specific binding of LPG and rapidly divide. After further 4-7 days, the parasites cease dividing and differentiate into infective metacyclic promastigotes, which have a structurally altered LPG that is incapable of binding to the mid gut wall (Sacks et al., 1995). They migrate to the foregut and esophagus, where they are suspended in the sandfly's saliva and are ready to be inoculated during the next blood meal (Schlein et al., 1992).

1.5.2 ***Leishmania life cycle in the mammalian host:*** In order to get into their host, *Leishmania* take advantage of the feeding habits of the vector. The sandfly rips up the epidermis and gains access to dermal capillaries, regurgitating parasites into the bite wound (Warburg & Schlein, 1986). Presence of multiple receptors allows the parasite easy access into macrophages and langerhans cells where the parasites transform into amastigotes. Langerhans cells are thought to provide a safe haven for the parasite by their failure to produce inducible nitric oxide synthase; iNOS (Moore & Matlashewski, 1994). More significantly, although parasites fail to replicate in langerhans cells, they are not rapidly killed and might save the host cells from apoptosis (Moore & Matlashewski, 1994). Otherwise, the sites of *Leishmania* infection are characterized by a marked increase in the number of macrophages because they are unable to migrate from these sites. In addition to parasite products and virulence factors, components in sandfly saliva suppress macrophage leishmanicidal activity, inhibits nitric oxide (NO) production (Hall & Titus, 1995; Bates & Rogers, 2004).



(A) (B)

Figure 1.7.1: Two stages of *Leishmania* parasite (**A**) Extracellular and motile form called promastigotes each bearing a flagellum. (**B**) Intracellular and non motile stage called amastigotes (small dots) as seen in Giemsa stained dab smear prepared from the spleen of *L. donovani* infected golden hamster.

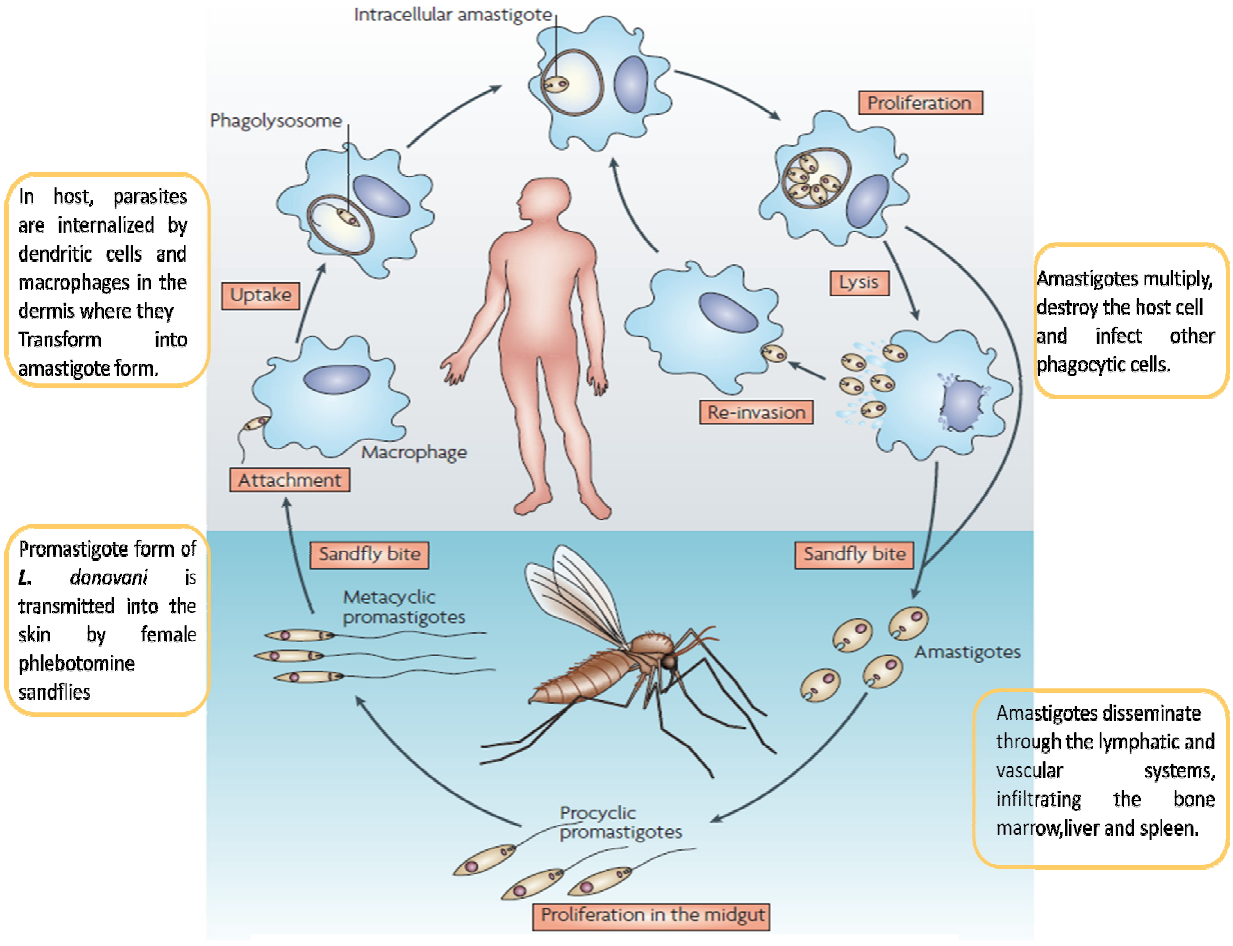


Figure 1.7.2. The life cycle of *Leishmania donovani* parasite. (Reproduced from Chappuis et al., 2007, Nature Rev Microbiol, 5:S7-15).

1.6. Host-parasite interactions

Protection of promastigotes from the killing and degradative activities of macrophages is provided by the surface structures LPG and gp63 on the metacyclic promastigote. By preferentially accessing macrophages via complement components CR3 and CR1, the promastigotes fail to trigger the macrophage respiratory burst (Brittingham & Mosser, 1996). LPG also transiently inhibits phagosome-endosome fusion (Desjardins & Descoteaux, 1997), scavenges oxygen radicals generated during the respiratory burst (Chan et al., 1989), inhibits protein kinase C activity (Giorgione et al., 1996) and suppresses macrophage iNOS expression and NO production (Proudfoot et al., 1996; Van Assche et al., 2011). gp63 has also been associated with suppression of the oxidative burst (Sorensen et al., 1994), and compelling evidence suggests that its protease activity protects the parasite from lysosomal cytolysis and degradation (Seay et al., 1996). Fusion of phagosome-lysosome takes place as the metacyclic transforms into the small ovoid amastigotes, and the parasites are able to survive and multiply within the acidic, hydrolase rich parasitophorous vacuole (Kaye & Scott, 2011). This transformation is associated with down regulation of LPG (McConville & Blackwell, 1991) and gp63 (Bahr et al., 1993) expression on the parasite surface.

Amastigotes rely on opsonization using the macrophage Fc receptor as indicated by studies on *L. major* (Guy & Belosevic, 1993) and *L. mexicana* (Peters et al., 1995) amastigotes. Evidently, promastigotes and amastigotes can enter host cells by multiple routes and this redundancy indicates that the route of entry is ultimately not the deciding factor in determining intracellular parasite survival (Mosser & Rosenthal, 1993).

1.7. Unusual features in *Leishmania* biology

Leishmania belongs to the order *Kinetoplastida* and have a unique organelle called the kinetoplast, an appendix of their single mitochondrion located near the basal body of the flagellum that contains a network of thousands of small interlocking circular DNAs. Kinetoplastids are among the most ancient eukaryotes, with rRNA lineages extending farther back than those of animals, plants, and even fungi (Fernandes et al., 1993; Beverley, 1996). Several fundamentally important biological phenomena in *Leishmania* include 'programmed' antigenic variation of surface glycoproteins (Bridgen et al., 1976), glycosylphosphatidylinositol anchors of membrane proteins (Ferguson & Cross, 1984), expansion and contraction of telomeric DNA repeats (Bernards et al., 1983), bent DNA helices (Marini et al., 1983), eukaryotic polycistronic transcription (Johnson et al., 1987), trans splicing of precursor RNAs (Walder et al., 1986), mitochondrial RNA editing (Benne et al., 1986), other unique organelles such as glycosomes and distinctive metabolic pathways. In addition, the many novel mechanisms are used by *Leishmania* and Trypanosomes to thwart immune defenses thrown at them by their mammalian hosts (Donelson et al., 1999). None of the protein encoding genes of *Leishmania* contain introns and most of these genes are initially transcribed into large polycistronic precursor RNAs that are cleaved into monocistronic mRNAs by trans splicing (Matthews et al., 1994).

In summary, evolutionarily ancient *Leishmania* and Trypanosomes are placed at the genetic border between prokaryotic and eukaryotic organisms. They share some features with prokaryotes (genes without introns, polycistronic transcription), other features with eukaryotes (pre mRNA splicing), and have some features found in neither

(protein encoding genes without promoters) making this organism a very interesting model.

1.8. *Leishmania* genome organization

The *Leishmani*a genome is a relatively small eukaryotic genome with an estimated size of $3.5X10^7$ bp. Old World *Leishmania* (*L. donovani* and *L. major* groups) have 36 chromosome pairs (0.28 to 1.8 Mb) (Wincker et al., 1996), whereas New World species have 34 or 35, with chromosomes 8+29 and 20+36 fused in the *L. mexicana* group and 20+34 in the *L. braziliensis* group (Britto et al., 1998) and possess repetitive telomeric sequences. The chromosomal organization of *Leishmania* is similar to many protozoan parasites; a compartmentalization into conserved core domains and polymorphic chromosome ends (Lanzer et al., 1995). In terms of structure and maintenance of chromosomal termini, *Leishmania* conforms to those described in other eukaryotes. Telomerase activity has been demonstrated in *Leishmania* with primer recognition and elongation properties similar to those of other eukaryotes (Cano et al., 1999). *Leishmania* were found to be more G/C rich (58%) than *T. brucei* (51%) or *T. cruzi* (44%) (Alonso et al., 1992) that might be a reflection of the more primitive nature of these organisms.

The genome sequence of *L. major* released in July, 2005 was obtained by shotgun sequencing that provided a framework for future comparative genomic studies (Ivens et al., 2005). The genome elucidated the full structural architecture of *Leishmania* chromosomes, which includes an unusual pattern of genes distributed in large directional clusters. In *L. major* genome, out of ~8379 genes, 310 are experimentally characterized,

the identity of 2610 genes is inferred from homology to other databases, 4674 genes code for conserved hypothetical proteins, 690 sequences are orphan meaning no predicted function can be attributed to them and 71 genes are termed as pseudogenes and have premature stop codons and/or frame shifts (Ivens et al., 2005). Subsequently, the genomes of *L. braziliensis* and *L. infantum* were described (Peacock et al., 2007). The *L. infantum* genome contains 8184 genes present on 36 chromosomes and *L. braziliensis* contains 8312 genes present on 35 chromosomes. The difference in chromosome number is due to the fusion of chromosomes 20 and 34 to make a single chromosome in *Leishmania braziliensis*. Sequencing of *L. donovani* BPK282 genome has been recently completed and the organism is reported to contain 8489 genes present on 36 chromosomes (Downing et al., 2011). Reports on other classes of genome diversity between *Leishmania* lines, such as structural polymorphisms within tandem arrays and the generation of episomes have been speculated to be contributing to substantial differences to gene expression (Dujardin, 2009; Leprohon et al., 2009a).

Comparison of the genomes of *L. major, L. infantum* and *L. braziliensis*: A striking background of conservation at the gene content level with almost complete synteny was observed in 3 *Leishmania* genomes. Divergence between the *Leishmania* species complexes is estimated to have occurred 15–50 million years ago (Lukes et al., 2007). Despite the differences in gene copy number within some of the major protein coding families described above, not a single chromosomal rearrangement has been identified between *L. major* and *L. infantum* across the whole genome, while *L. braziliensis* has only a few possible sequence rearrangements (Peacock et al., 2007). Equally surprising, from the total content of 8,300 genes in each species, only 200 can be identified as

differentially distributed between the three genomes. The most divergent, *L. braziliensis*, possesses 47 genes that are absent from the other two species. In comparison, *L. major* has 27 species specific genes while *L. infantum* has only five. Comparison of the three *Leishmania* genomes has revealed that gene variation is evenly distributed across the genome (Peacock et al., 2007). These degenerate sequences have in-frame stop codons and frame shifts, generating truncated open reading frames that are presumably not translated. Acquisition of novel genes in this way may be a mechanism for environmental adaptation to promote survival (Peacock et al., 2007). Despite its chromosomal plasticity (Martinez-Calvillo et al., 2005), the incredible conservation of synteny revealed by comparative genomic analyses of these three species suggests that the *Leishmania* genome is highly stable and has not undergone major genomic rearrangements during speciation. One contributing factor to this stability could be a lack of mobile DNA elements, as originally demonstrated in the *L. major* genome and verified in *L. infantum* (Bringaud, 2005). The comparative sequencing project has revealed some surprising observations, however, one of the most striking being the presence of transposable elements in *L. braziliensis* (Peacock et al., 2007).

1.9. Immunology of Leishmaniasis

In order to survive within macrophages, *Leishmania* must achieve silent infection, avoiding activating the antimicrobial respiratory burst and evading immune responses (Bogdan & Rollinghoff, 1998). Antileishmanial immunity is mediated via both innate (macrophages, neutrophils) and adaptive (B cells, T cells and DCs) type of immune responses. Macrophages play a pivotal role in *Leishmania* infection. A successful treatment of all the forms of Leishmaniasis depends on efficient elimination of parasites

by activated macrophages. Paradoxically, *Leishmania* utilises their phagocytic function as a strategy for internalization and replication within the phagolysosomes (Reiner & Locksley, 1995) Thus, macrophages act as both the host cells and effector cells that kill the parasites. Internalization of *Leishmania* by macrophages leads to the production of proinflammatory cytokines and parasite killing. Induction of a Th1 immune response is associated with clearance of *Leishmania* infection, whilst a Th2 response leads to persistence. Th1 cells secrete protective cytokine IFN-γ (Gamma-interferon) and interleukin-2 (IL-2) and execute CMI responses (Delayed hypersensitivity and macrophage activation), where as Th2 cells produce disease exacerbative cytokines IL-4, IL-10 and IL-13 and assist in antibody production for humoral immunity (Reiner & Locksley, 1995; Suffia et al., 2005). The regulatory cytokines TGF-β and IL-10 have also been suggested as important as immunosuppressive signaling molecules in *Leishmania* infections (Olivier et al., 2003). A subversive activity of *Leishmania* parasites in this process is the inhibition of IL-12 production, which is necessary for the leishmanicial activity of macrophages (Ahuja et al., 1999), as it leads to upregulation of iNOS, NO and IFN-γ. Production of cytokines results in the recruitment of other proinflammatory cells (neutrophils, mast cells and macrophages) to the site of infection. In particular, neutrophils are among the first cells recruited to the site of infection and are thought to participate in the containment of *Leishmania* parasites within an hour of infection (Belkaid et al., 2000).

A similar Th1/Th2 dichotomy in the cytokine response to *L. donovani* infection in murine and human system has also been reported (Karp et al., 1993; Sundar et al., 1997). Cytotoxic CD8+ T cells also play a host protective role, and are required for the effective

clearance of parasites and the generation of memory responses. Interestingly, 80 to 90% of human infections are subclinical or asymptomatic, and this asymptomatic infection is associated with strong cell mediated immunity. Only a small percentage of infected individuals develop severe disease, and patients who recover from VL display resistance to reinfection. This suggests the development of clinical immunity and provides a biological rationale for the development of VL vaccines that impart a strong cellular immunity.

Active VL disease is characterized by the marked elevation of humoral immune response i.e. by the production of plenty of specific as well as nonspecific antibodies (Ghosh et al., 2001a). Antibody titers, primarily IgG rise sharply during VL, but the antibodies so generated, are apparently not protective (Evans et al., 1990). The enormous increase in the serum immunoglobulin levels in active KA is due to polyclonal activation of immunoglobulin producing cells leading to increased biosynthesis of IgG and to a lesser extent, IgM. Most of the antibodies produced during infection are not parasite specific (Evans et al., 1990), but the hyper gammaglobulinemia may have diagnostic value. Although strong humoral responses are induced by VL infection, antibodies play no role in protection and are often associated with disease exacerbation.

1.10. VL/HIV co-infections

VL and human immunodeficiency virus (HIV) co-infection has emerged as a serious disease pattern (Desjeux et al., 2001; Desjeux & Alvar, 2003; Cota et al., 2011). HIV infection increases the risk of developing VL by 100 to 2,320 times in endemic areas (Gradoni et al., 1996; Alvar et al., 1997) and, on the other hand, VL promotes the clinical

progression of HIV disease and the development of AIDS defining conditions (Alvar et al., 2008). Both infections switch the predominantly cellular immunological response from Th1 to Th2 through complex cytokine mediated mechanisms leading to a synergistic detrimental effect on CMI (Olivier et al., 2003; Sinha et al., 2006). To date, VL/HIV coinfection has been reported from 31 countries, with most of the cases from Southern Europe, where 25–70% of adult patients with VL are co-infected with HIV (Desjeux, 2001). The poor therapeutic outcome, the high rate of relapse, the polyparasitic nature of VL in HIV infected persons, as well as the atypical manifestations of the disease and the impaired access to health care resources make HIV infected individuals prone to enlarge the number of human reservoirs (Molina et al., 2007). This concern is of utmost importance in Asia, where HIV and *Leishmania* coinfections are increasingly being reported in countries that are also facing parasite resistance to antimonial drugs (Sundar, 2001). Since AIDS epidemic is looming large on the horizon of new millennium in India (Sinha et al., 2006), the state of Bihar needs to be looked seriously for VL/HIV co-infections. From a global epidemiologic viewpoint, two parallel trends are alarming; the ruralization of the HIV pandemic and the urbanization and spread of VL (Rabello et al., 2003). The reported global incidence of co-infection is likely underestimated because VL has not been included in the list of AIDS related opportunistic infection in all endemic areas (WHO, 2009). The co-infection is now becoming proportionately more prominent in areas with poor access to anti retrovirals, such as Africa. In areas where it is available, Highly Active Antiretroviral Therapy (HAART) has changed the course of the HIV/AIDS epidemic and the outcome of associated opportunistic infections. However,

evidence of relapse rate reduction in patients using HAART is conflicting (Jimenez-Exposito et al., 1999).

1.11. Control strategies for Visceral Leishmaniasis

Efficient VL case management based on early diagnosis and treatment is the key to limit morbidity and prevent mortality (Matlashewski et al., 2011). Effective treatment of patients is also a measure to control reservoir and transmission in anthroponotic foci, particularly for cases of PKDL, which are thought to act as a long term reservoir of the disease. Indian government started Leishmaniasis elimination programme in 2001 and presently, Governments of India, Nepal and Bangladesh have launched a regional VL elimination programme for reducing the annual incidence from 24.9 (Singh et al., 2010) to less than 1 case per 10,000 individuals by 2015 (Chappuis et al., 2007). Elimination of kala azar from the Indian sub-continent is based on three pronged approach; early diagnosis, effective and implementable treatment, and effective vector-control.

1.12. Vector control

Leishmaniasis is transmitted by the *Phlebotomus* spp. in the old world and *Lutzomyia spp.* in the new world. *P. argentipes* is the proved vector of KA in India (Kishore et al., 2006; Swaminath et al., 2006). Of the 500 known Phlebotomine species, only some 30 of them have been positively identified as vectors of the disease. Sandflies are very small in size (< 3.5 mm) (Fig. 1.8) and may be hard to see.

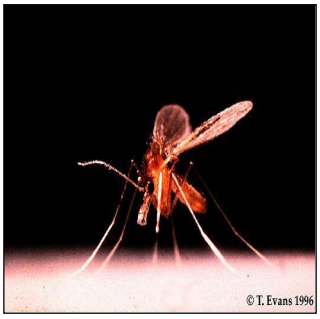


Figure 1.8: ***Phlebotomus* sand fly** The vector host of *Leishmania* parasite.

Sandflies are susceptible to the same insecticides as *Anopheles* mosquitoes, the malaria vector. Residual insecticide spraying of houses and animal shelters was shown to be efficacious in India (Kaul et al., 1994), where the vector (*P. argentipes*) is restricted to areas in and around the home. Following the large scale antimalarial insecticide dichloro diphenyl trichloroethane (DDT)) spraying campaigns that were implemented in the 1950s, VL almost completely disappeared from the Indian subcontinent. Unfortunately, the disease quickly reemerged when these spraying campaigns were discontinued. Resistance of *P. argentipes* to DDT remains limited, but has been reported in Bihar (Picado et al., 2010). The use of insecticide treated bednets (ITNs) could prevent VL and other vector borne diseases. Despite low usage, the mass distribution of ITNs in Sudan was accompanied by a 27% reduction in the incidence of VL in an observational study (Ritmeijer et al., 2007). However, recent report provided evidence that ITNs have a limited effect on sandfly exposure in VL endemic communities in India and Nepal (Gidwani et al., 2011).

1.13. Diagnosis of Visceral Leishmaniasis

Laboratory diagnosis of Leishmaniasis by direct visualization of amastigotes in clinical specimens is the diagnostic gold standard in regions where tissue aspiration is feasible and microscopy and technical skill are available. Diagnostic sensitivity for splenic, bone marrow, and lymph node aspirate smears is >95%, 55–97%, and 60%, respectively (Siddig et al., 1988). Elsewhere, serum antileishmanial immunoglobulin G in high titre is the diagnostic standard, primarily with direct agglutination tests or other laboratory based serological assays (Herwaldt, 1999; Desjeux, 2004). Currently, rK39 strip test and Direct Agglutination Test (DAT) are widely used at field level for the

primary diagnosis of VL (Fig 1.9), while PCR assays are used in referral labs. In symptomatic patients, anti-K39 strip test sensitivity is 90–100% (Sundar & Rai, 2002c; Boelaert et al., 2008), while specificity might vary by region. This test can safely substitute for invasive diagnostic procedures in VL and is also useful for PKDL. The rK39 strip tests perform well in the Indian subcontinent, but further development and evaluation is required in East Africa, with specific attention to the diagnostic accuracy in HIV co-infected patients. Testing urine for leishmanial antigen or antibody is a new approach (Islam et al., 2004; Sundar et al., 2005). Various immunodiagnostic tests include antibody detection, complement fixation test, immunodiffusion test, indirect hemagglutination, IFA test, DAT, ELISA with rK39 antigen and rapid strip test with rK39. In addition, antigen detection test called KATEX is 68-100% sensitive (Chappuis et al., 2007).

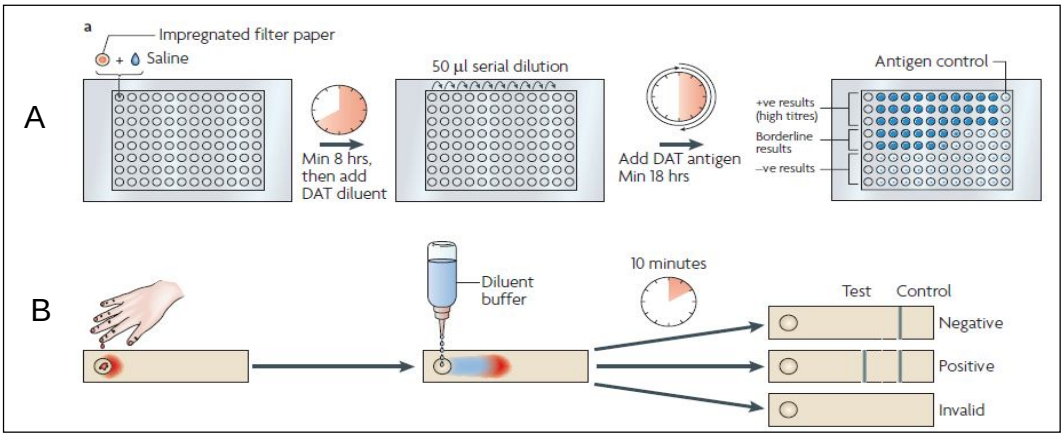


Figure 1.9: Serological tests for visceral leishmaniasis. (A) The direct agglutination test. (B) The rK39 immunochromatographic test strip. (Reproducedcv 0020 from Chappuis et al., 2007, Nature Rev Microbiol, 5:S7-15).

Molecular approaches have become increasingly relevant due to remarkable sensitivity, specificity and flexibility in choice of samples. DNA detection by PCR with LDI primer

using blood, bone marrow and skin are currently under use (Salotra et al., 2001). Different DNA sequences in the genome of *Leishmania* have been documented in diagnosis and prognosis of VL (Schallig & Oskam, 2002). Quantitative PCR is a highly sensitive and specific tool used in referral labs for detection/assessment of parasite load in VL patients. Remarkable sensitivity and specificity and ability to quantify parasite load pre and post treatment indicated its robust potential in VL diagnosis and monitoring of the treatment (Verma et al., 2010). The method displays potential to provide threshold for distinguishing asymptomatics in endemic areas.

Currently, improvement in VL diagnostics is required for successful decentralized (point of care) testing in field conditions and to detect VL-HIV co-infection. Techniques such as loop mediated isothermal amplification (LAMP) offer a reliable molecular diagnostic method for field application (Takagi et al., 2009; Adams et al., 2010). The diagnosis based on bioanalytics/biosensors promise as frontiers for point of care VL detection after adequate standardization. In addition to the VL confirmation, the available diagnostic markers should also serve as a test of cure after therapy, marker for asymptomatic infections and detect drug resistance.

1.14. Vaccines against Visceral Leishmaniasis

The development of a vaccine against Leishmaniasis is a long term goal in both human and veterinary medicine. In the past decade, various subunit and DNA antigens have been identified as potential vaccine candidates in experimental animals but none have so far been approved for human use (Evans & Kedzierski, 2012). To date there is no vaccine against VL in routine use anywhere in the world though several vaccine

preparations are in more or less advanced stages of testing. Unfortunately, the development of vaccines has been hampered by significant antigenic diversity and the fact that the parasites have a digenetic life cycle in at least two hosts.

Numerous preparations of killed parasites were tested over decades as first generation vaccines, either alone or in combination with a variety of different adjuvants. However, none have demonstrated sufficient efficacy as a prophylactic vaccine to be used in widespread control programmes (Noazin et al., 2009). Interestingly, killed parasite vaccines using an alum precipitated autoclaved *L. major* (ALM) given with a BCG adjuvant have shown promise as vaccines for VL and PKDL (Khalil et al., 2005). When given to patients with persistent PKDL in combination with antimonial therapy, this vaccine showed enhanced cure rates and lower incidence of relapse as compared to antimonial treatment alone (Musa et al., 2008).

The development of second generation vaccines for *Leishmania* has included recombinant proteins, polyproteins, DNA vaccines, liposomal formulation, and dendritic cell vaccine delivery systems. Immunization with the *L. donovani* A2 cysteine proteinase delivered as recombinant protein or as DNA also afforded protection against experimental challenge infection (Ghosh et al., 2001b). Other antigens tested include amastigote cysteine proteases (CPs) (Rafati et al., 2006), kinetoplastid membrane protein-11 (KMP-11) (Basu et al., 2005), amastigote LCR1 (Streit et al., 2000), leishmanial antigen ORFF (Tewary et al., 2005), and NH36, a main component of the fucose-mannose ligand (Aguilar et al., 2005). Apart from defined single molecules, multicomponent vaccines have been shown to protect against VL in experimental infection systems. One multicomponent vaccine, Leish-111f, has been assessed in clinical

trials. Leish-111f is a single polyprotein composed of three molecules fused in tandem: the *L. major* homologue of eukaryotic thiol specific antioxidant (TSA), the *L. major* stress inducible protein-1 (LmSTI1), and the *L. braziliensis* elongation and initiation factor (LeIF) (Coler & Reed, 2005). A recent small scale clinical trial in a *L. donovani* endemic area showed Leish-F1-MPL-SE was safe and well tolerated in people with and without prior VL exposure and induced strong antigen specific T cell responses (Chakravarty et al., 2011).

Vaccination strategies using live attenuated *Leishmania* parasites are attractive as they closely mimic the natural course of infection and may elicit clinically protective immune responses. The use of live attenuated vaccines provides a promising vaccination strategy for VL; however safety issues regarding the use of genetically attenuated parasites as vaccines still need to be addressed. A loss of centrin from *L. donovani* parasites did not affect the growth of promastigote forms, but null mutants were unable to survive as axenic amastigotes or in human macrophages *in vitro* (Selvapandiyan et al., 2006) while lack of biopterin transporter (BT1) showed decrease in infectivity (Papadopoulou et al., 2002). Other approaches to developing live attenuated parasites as VL vaccines have utilised nonpathogenic *Leishmania* species, an approach comparable to the use of BCG as a vaccine against *Mycobacterium tuberculosis* infection (Azizi et al., 2009). A continuing synergy between molecular and immunological approaches to the development of VL vaccines will accelerate development of the next generation of therapeutics.

1.15. Treatment of Visceral Leishmaniasis

Treatment of VL is complex and difficult. There is a growing limitation in the availability of chemotherapeutic strategies, and emergence of resistant strains of *L. donovani* has further aggravated the crisis (Mondal et al., 2010). The drugs currently recommended for the treatment of VL include the Pentavalent Antimonials viz. Sodium Antimony Gluconate (SAG), Amphotericin B and its lipid formulations, Miltefosine and recently, Paromomycin (Fig 1.10).

1.15.1 Pentavalent antimonials [Sb(V)]

Worldwide, SbV has been the first line treatment for VL for the last 70 years. These are available as branded products, meglumine antimoniate (Glucantime) and sodium stiboguconate (Pentostam) (Fig. 1.12) and in the generic form, Sodium Antimony Gluconate (Albert David Ltd., Kolkata). The major concern for antimonials is that they exert toxic effects like nausea, abdominal pain, pancreatitis and cardiotoxicity. Moreover, the loss of antimonial efficacy in North Bihar was a clinical disaster leaving 45% of the world's VL patients in a precarious situation as there was no immediate clear cut substitute treatment available (Sundar & Chatterjee, 2006). In Bihar, since the mid 1990s, only 36–69% VL cases showed cure demonstrating the progressive downfall of SbV treatment (Sundar et al., 2000; 2002) rendering the drug useless for routine VL therapy. This calamity created awareness in the scientific community on the neglected status of VL treatment and slowly rekindled efforts to generate new treatment alternatives or refinements.

Mechanism of action: The mode of action of antimonials has not been clearly elucidated yet. It is thought that pentavalent antimonials [Sb(V)] are prodrugs that require *in vivo* reduction to the leishmanicidal trivalent form [Sb(III)]. The site (host macrophage, amastigote or both) and the mechanism of reduction remain unclear (Zhou et al., 2004). Previous work suggested that antimonials inhibit macromolecular biosynthesis in amastigotes, possibly due to the inhibition of glycolysis and fatty acid beta oxidation (Berman et al., 1987). Antimony induces efflux of the intracellular trypanothione, and also inhibits the enzyme trypanothione reductase (Wyllie et al., 2011). These two mechanisms compromise the thiol redox potential of the cell and lead to the accumulation of reactive oxygen species (ROS). SbV binds to the ribose moiety and forms stable complexes with adenine nucleosides, which act as inhibitors of *Leishmania* purine transporters (Demicheli et al., 2002). Sb(III) and Sb(V) have been found to induce apoptosis and death by DNA fragmentation and externalisation of phosphatidylserine (Sudhandiran & Shaha, 2003).

Mechanism of Antimony resistance: Several mechanisms have been proposed to explain *Leishmania* resistance to antimonials: diminished conversion of Sb(V) to the biologically active Sb(III), decreased uptake of Sb(III) by decreasing the expression of transporters which mediate the uptake of Sb(III), and increased efflux/sequestration of Sb(III) in conjugation with thiols (Croft et al., 2006; Ashutosh et al., 2007). Recent results suggest that enhanced antioxidant defenses, through overexpression of Tryparedoxin peroxidase (TryP) may well be a key factor in the mechanisms of clinical resistance to antimonial drugs (Wyllie et al., 2008). Recent study demonstrated the role of Histone H2A in antimony resistance (Singh et al., 2010). Overall information on antimony resistance till

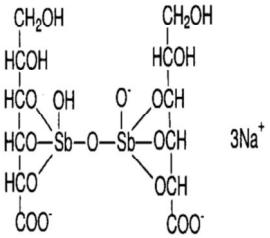
date indicates that several mechanisms may coexist in the same cell and that different mechanisms may operate in field isolates compared with laboratory generated resistant parasites (Croft et al., 2006). Recent study demonstrated that membrane composition is drastically modified in drug resistant parasites compared with drug sensitive parasites (t'Kindt et al., 2011).

1.15.2. Amphotericin B

Amphotericin B (AmB) is an antifungal macrolide antibiotic isolated from *Streptomyces nodosus* in 1956. Its antileishmanial activity was first shown in the early 1960s attributed to its selective affinity for ergosterol vis-a-vis cholesterol (Ramos et al., 1996). AmB, at a dose of 0.75-1.0 mg/kg for 15 to 20 infusions either daily or on alternate days, has consistently produced cure rates of about 97% and is now the drug of choice in North Bihar (Sundar & Rai, 2002). AmB treatment requires in-patient care in a relatively well equipped hospital for a period of one month. Thus, while AmB is the drug of choice in referral hospitals in India, it is unsuitable for use in most remote areas where such facilities are lacking. The use of this drug has been restricted by its toxic side effects (Wasan et al., 2009).

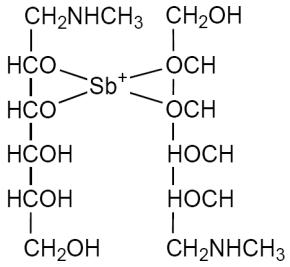
Mechanism of Action: AmB has less affinity for cholesterol, predominant in the plasma membrane of mammalian cells, than for ergosterol, predominant in fungi and Trypanosomatids. AmB acts on parasite membrane sterols and inserts in ergosterol resulting in an increase in permeability and parasite death (Romero et al., 2009). AmB resistant promastigotes exhibited decreased drug uptake, increased efflux and increased membrane fluidity of the AmB resistant parasite (Mbongo et al., 1998).

(A)



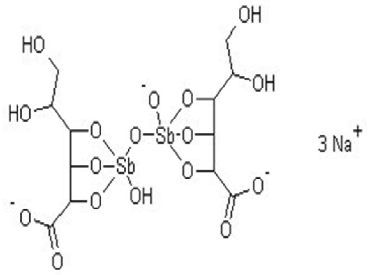
Sodium antimony gluconate

(B)



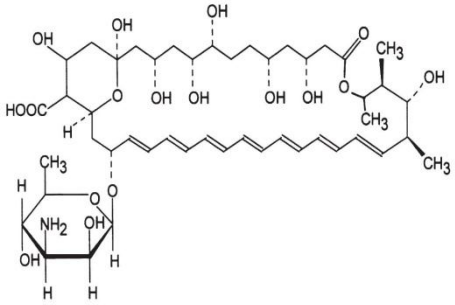
Meglumine antimonite

(C)



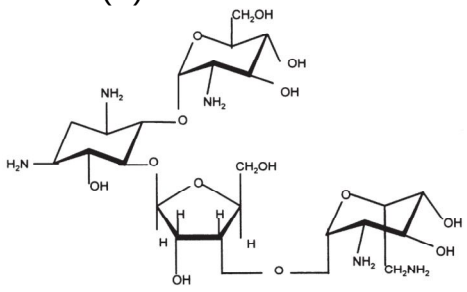
Sodium stibo gluconate

(D)



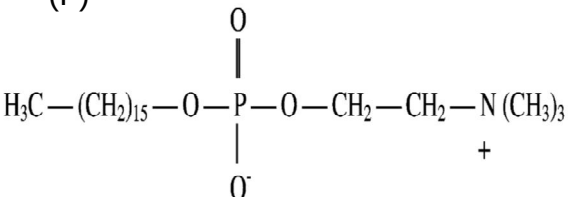
Amphotericin B

(E)



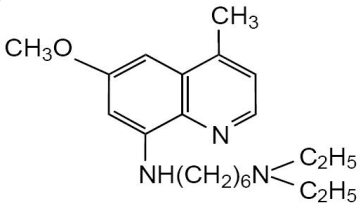
Paromomycin sulphate

(F)



Miltefosine

(G)



Sitamaquine (WR6026)

Figure 1.10: Antileishmanial drugs currently available for VL treatment or under various stages of clinical trials. (A-C): Different compounds of pentavalent antimonial drugs. (D) Amphotericin B is a polyene macrolide drug. (E) Paromomycin sulphate is an aminoglycoside antibiotic (F) Miltefosine is an alkyl phosphocholine drug (hexadecyl phosphocholine). (G) Sitamaquine is an 8-aminoquinoline compound.

The lipid formulations of AmB have an upper edge as they produce distinctly lower toxicities, notably the absence of nephrotoxicity and significantly lower infusion reactions. With this preferential pricing, single dose AmBisome treatment (10 mg/kg) is cost effective compared with conventional AmB treatment (Sundar et al., 2008). Amphomul (Bharat Serums and Vaccines, India), a new AmB emulsion, has proved highly effective and safe in a Phase II study of VL patients in three different short course dosing regimens (Murray, 2010).

1.15.3. Miltefosine (Hexadecylphosphocholine)

Miltefosine (MIL), initially developed as an anticancer drug, is the first effective oral treatment of VL. Its antileishmanial activity was initially discovered in the mid 1980s and since then its efficacy has been demonstrated in several *in vitro* and *in vivo* experimental models (Croft et al., 2003). Since 2002, MIL has been used successfully in India for oral treatment of VL (Croft et al., 2006; Berman, 2008), although its use is compromised by teratogenicity (Dorlo et al., 2008) and gastrointestinal side effects such as anorexia, nausea, vomiting and diarrhea (Sundar et al., 2002). Efficacy of MIL has also been established for the treatment of PKDL in India (Sundar et al., 2006; Ramesh et al., 2011).

Mechanism of Action: Several molecular targets in Trypanosomatids have been suggested for MIL; alkyl phospholipid metabolism, glycosyl phosphatidylinositol biosynthesis and the interference with ether lipid remodelling through the inhibition of alkyl lyso phosphatidylcholine specific acyltransferase (Lux et al., 2000). It has also been

reported that miltefosine induces apoptosis like death (Paris et al., 2004; Verma & Dey, 2004).

Mechanism of resistance: Impairment in activity of P-Type ATPase transporters, LdMT–LdRos3 dependent flippase machinery has been reported in experimental MIL resistance (Perez Victoria et al., 2006a). A gene from *L. infantum*, upon over expression, was shown to confer protection not only against MIL, but also against Sb(III) (Choudhury et al., 2008). There are no reported human cases of MIL resistant Leishmaniasis till date. However, the prolonged half-life of MIL and the ability to generate drug resistant lines suggest a rapid development of MIL resistance (Castanys-Muñoz et al., 2008; Sundar & Olliaro, 2007) and relapse when used as a monotherapy, especially in incomplete courses. Moreover, phase IV trial of MIL in India suggested doubling of the relapse and failure rate compared to phase III trials (Sundar & Rai, 2005). Relapse of VL after MIL treatment have been reported (Sundar et al., 2006; Pandey et al., 2009). Development of PKDL after successful treatment of VL with MIL was recently reported in patients from India (Das et al., 2009; Kumar et al., 2009a). These reports warrant the studies on understanding the mechanism of resistance to this drug in order to prevent the emergence of resistance to this valuable class of drug.

1.15.4. Paromomycin (Aminosidine)

Paromomycin (PMM) is an aminoglycoside antibiotic, first isolated from *Streptomyces krestomuceticus* in the 1950s. It remained neglected as antileishmanial drug until the 1980s when topical formulations were found to be effective in CL and a parenteral formulation for VL was also developed. One World Health, the Bill and

Melinda Gates Foundation, Gland Pharma Limited, IDA Solutions and WHO/TDR partnered to develop PMM injection as a public health tool to be sold on a not for profit basis at a very low price. In phase III clinical trial, 94.6% patients treated with its injection were cured of VL. PMM injection was approved on August 31, 2006 for treatment of VL in India (Sundar et al., 2007). Recently, PMM has shown an efficacy of 94.2% in Phase IV clinical trials in India by iOWH (Sinha et al., 2011).

Mechanism of Action: Ribosomes could be the primary targets of PMM. In *L. donovani*, PMM promoted ribosomal subunit association of both cytoplasmic and mitochondrial forms, following low $Mg2^{+}$ concentration induced dissociation (Maarouf et al., 1995). PMM also induces respiratory dysfunction and appears to have other effects such as altering membrane fluidity and membrane permeability (Maarouf et al., 1997). Recent comparative proteomic analysis of the wild type and the PMM resistant strains showed upregulation of the ribosomal proteins, glycolytic enzymes and vesicular trafficking proteins in the PMM resistant parasite (Chawla et al., 2011).

1.15.5. Sitamaquine (WR 6026)

Sitamaquine (SIT), an orally active 8-aminoquinoline analog, was originally developed as WR6026 by the Walter Reed Army Institute of Research in collaboration with GlaxoSmithKline. Its antileishmanial activity was first identified in 1970s (http:// www.wrair.army.mil). It has shown good efficacy in Phase II clinical trials for VL (Jha et al., 2005).Toxicity appears to be relatively mild as it causes mild methemoglobinemia. Additional adverse events include nephrotoxicity (nephritis), abdominal pain and headache, mostly observable in patients receiving higher doses. While SIT may not be

effective as a monotherapy for VL, this oral agent may be developed as a part of combination therapy (Dietze et al., 2001).

Mechanism of Action: SIT is rapidly metabolized, forming desethyl and 4-CH_2OH derivatives, which might be responsible for its activity. The compound causes alkalisation of acidocalcisomes and collapse in mitochondrial membrane potential (Vercesi et al., 2000). It has been shown that its antileishmanial action is unrelated to its level of accumulation in acidocalcisomes (López-Martín et al., 2008). Recent study demonstrated that SIT acts by inhibition of respiratory chain complex II, which in turn triggers oxidative stress and finally leads to an apoptosis like death of *Leishmania* parasite (Carvalho et al., 2011).

1.15.6 Combination therapy

To prevent the development of resistance, combination therapy with two or more antileishmanial drugs appears to be an important approach for the treatment of VL. First, combinations of potent drugs should include one with a short half-life to rapidly bring down the parasite burden to prevent development of resistance, and the second drug should have a long half-life, which will kill the remaining parasites (Sundar et al., 2006). A second strategy would be the use of two drugs with different modes of action to reduce the chance of selection and survival of the resistant mutants (van Griensven et al., 2010). The third approach would be the use of two relatively weak drugs with synergistic or additive effects without drug interactions. This would shorten treatment duration and hospital stay, reduce treatment cost, prevent the emergence of drug resistant parasites, potentially decrease toxicity with lower drug doses, increase the spectrum of activity of

the drugs (Croft et al., 2006) and improve compliance. A combination therapy needs to be evaluated for safety and optimized for either concomitant or sequential administration of component drugs. The combinations that have either been carried out or are ongoing for the treatment of VL include SSG plus paromomycin (Jha, 2006; Melaku et al., 2007), Liposomal AmB (AmBisome) plus Miltefosine (WHO, 2009). Recently efficacy of the combinations: single injection of 5 mg/kg L AmB and 7 day- 50 mg oral Miltefosine or single 10 day- 11 mg/kg intramuscular Paromomycin; or 10 days each of Miltefosine and Paromomycin were demonstrated. All three combination treatments were found to be effective and non inferior to standard AmB treatment, and they were less toxic (particularly for the kidneys) and better tolerated than AmB. Reduced probability of development of resistant parasites, decreased duration of therapy, high efficiency and safety are advantages offered by combination regimens (van Griensven et al., 2010, Sundar et al., 2011).

1.15.7. Future treatment options

The existing drugs used to treat VL were all developed for other indications (Matlashewski et al., 2011). Research into potential drugs continues, with a more rational approach to drug design being taken (Davis et al., 2004; Seifert, 2011). Fundamental research into *Leishmaina* specific drug targets is one of the most promising areas. A number of potential antileishmanial drugs have been identified and are at various stages of development. Using the WHO/Tropical Diseases Research target database, targets have been prioritized for their druggable properties to progress to the screening stage. Different molecules screened by DNDi for the treatment of VL that are presently in the preclinical stage include buparvaquone, alternative formulations of AmB, nitroimidazole

and eight aminoquinolines. Research has shown that compounds have promising antileishmanial activity. Preliminary screening of cysteine protease inhibitors and pyridones by GlaxoSmithKline and DNDi has shown promising results. Other proposed drug targets include the metabolism of trypanothione and sterol biosynthesis enzymes and protein kinases (Croft & Coombs, 2003). The recent availability of the genomes of three *Leishmania* species and powerful bioinformatic tools has identified *Leishmania* specific genes, making them attractive potential drug targets (Ivens et al., 2005; Peacock et al., 2007).

Efforts are being made to search for natural antileishmanial compounds with high specificity and low toxicity. Many natural products have been investigated for their antiprotozoal properties *in vitro* (Dube et al., 2007; Kossuga et al., 2008; Singh et al., 2008). Marine organisms, due to unique environmental conditions, synthesize unique compounds that act as chemical defenses. Marine compounds offer an unprecedented opportunity for their pharmacological exploration and, hence, have received great attention during recent years (Faulkner, 2001). Secondary metabolites produced in marine organisms could be the source of bioactive substance and useful in modeling compounds for drugs (Haefner, 2003).

As early branching eukaryotes, the organization of their cells show extensive differences from that of mammalian cells, suggesting that there will be ample basis for identifying novel proteins, structures and processes that may exploitable as drug targets (Barrett et al., 1999).

Aims and Objectives

The aim of the study is to determine the natural susceptibility of the Indian *Leishmania donovani* field isolates from kala azar (KA) patients towards current/new antileishmanial drugs and to understand the mechanism of resistance towards new oral antileishmanial drug, Miltefosine (MIL). Genetic determinants of MIL resistance in KA will be explored by microarray technology that will simultaneously identify constellation of genes that exhibit altered expression in MIL resistant parasite. These gene products are most likely to provide an understanding of the MIL resistance mechanism, define targets to counteract MIL resistance and provide potential biomarkers of MIL resistance in KA.

SPECIFIC OBJECTIVES

1. *In vitro* assessment of sensitivity of *L. donovani* clinical isolates from KA patients towards current antileishmanial drugs: Miltefosine and AmphotericinB.
2. *In vitro* assessment of sensitivity of KA isolates towards new/potential antileishmanial drugs: Paromomycin, Sitamaquine and Marine extracts.
3. Generation and characterization of Miltefosine resistant *L.donovani* parasite.
4. Differential gene expression profiling of Miltefosine sensitive and resistant parasite using genomic microarray technique and analysis of interacting genes/ pathways involved in Miltefosine resistance.

1. *In vitro* assessment of sensitivity of *Leishmania donovani* clinical isolates from kala azar patients towards current antileishmanial drugs: Miltefosine and AmphotericinB.

Our previous studies on drug sensitivity status towards SAG in *L. donovani* parasites obtained from bone marrow aspirates of the KA patients revealed a good correlation between *in vitro* susceptibility and clinical response (Singh et al., 2006). We will therefore evaluate the *in vitro* susceptibility of field isolates from Indian KA patients (Both SAG responsive and nonresponsive) towards the alternate drug, AmphotericinB (AmB) and a recently introduced oral antileishmanial drug, Miltefosine (MIL), to understand the inherent susceptibility of the prevailing parasite population towards these drugs. In view of the reports on impairment in activity of P-Type ATPase transporters, LdMT–LdRos3-dependent flippase machinery in experimental MIL resistance, we will further evaluate the mRNA expression of LdMT and LdRos3 in the field isolates and determine any correlation with MIL sensitivity.

***2. In vitro* assessment of sensitivity of KA isolates towards new/ potential antileishmanial drugs: Paromomycin, Sitamaquine and Marine extracts**.

The sensitivity of KA isolates towards paromomycin and sitamaquine, drugs in successful clinical trials will be determined to obtain a baseline data on the natural susceptibility of the field isolates previously unexposed to these drugs. In order to understand how these drugs mount their cytotoxic effect on the intracellular parasites, the effect of paromomycin/sitamaquine treatment on the release of nitric oxide (NO) by *L. donovani* infected macrophages will be evaluated. Further, in an effort to discover safe,

effective and affordable treatment options for VL, 25 marine extracts will be evaluated for their potential antileishmanial activity.

3. Generation and characterization of Miltefosine resistant *L.donovani* parasite.

To investigate the antileishmanial mechanism of action and resistance of MIL, a stable *L.donovani* parasite line resistant to MIL will be generated experimentally. Based on the MIL sensitivity profile of KA field isolates, an *L donovani* isolate exhibiting higher tolerance to MIL will be selected and adapted to grow stepwise under increasing MIL pressure. This lab generated MIL resistant parasite will be characterized based on the growth profile, altered MIL sensitivity, *in vitro* infectivity, cross resistance to other antileishmanial drugs, and expression analysis of reported markers of MIL resistance[LdMT (P-type ATPase transporter at plasma membrane) and LdRos3 (its beta subunit)] at transcript level. This will provide us with a well characterized MIL resistant parasite that can be employed as a tool for studying the differential gene expression in Miltefosine resistant parasite.

4. Differential gene expression profiling of Miltefosine sensitive and resistant parasite using genomic microarray technique and analysis of interacting genes/ pathways involved in Miltefosine resistance

The mechanisms and related biological pathways that contribute to MIL resistance in the parasite are relatively poorly understood. The biochemical changes responsible for resistance of parasite towards MIL, are most likely the result of programmed changes in their gene expression. In recent times, gene expression microarray has become well established technology by which the expression of hundreds of genes can be measured simultaneously and provide a global genetic perspective on complex biological processes

like drug resistance. Transcriptome profiling of the MIL resistant *L.donovani* will therefore be carried out using whole genome *Leishmania* oligonucleotide array. Differential expression profiling of MIL sensitive/resistant parasite will help to identify key genes which may be regulatory for susceptibility or resistance to MIL. A set of differentially expressed genes will be analyzed by real time PCR to validate the results obtained from microarray analysis. The altered genes will be further analyzed for their involvement in specific pathways related to metabolism, cellular organization and biogenesis, transport etc that will give an insight into the mode of action and possible mechanism of resistance to MIL and will identify potential targets to counter the resistance mechanism.

In vitro susceptibility of Leishmania donovani field isolates to Miltefosine and Amphotericin B: correlation with SAG susceptibility and implications in field settings

Introduction

Protozoan parasites of *Leishmania* species are causative agents of disease ranging from cutaneous lesions to fatal visceral infections. A significant impact of *Leishmania* on human populations can be judged from 12 million affected cases spread across 88 countries (WHO, 2008). India is one of the major sufferers of visceral leishmaniasis (VL) or kala azar (KA) caused by *L. donovani,* sharing almost 50% of global burden of VL (Desjeux, 2001). In the absence of vaccine, chemotherapy is the only choice for control of the disease. However, the arsenal of antileishmanial drugs is limited due to variation in efficacy and emergence of drug resistance. Today, of all regions, Bihar is facing the most immediate public health problem as several districts in this region are troubled with the highest number (more than 60%) of non-responsive cases towards traditional antimonial therapy (Sundar, 2001). Anthroponotic transmission of the parasite further increases the chances for the fast generation and spreading of drug resistant parasites (Singh et al., 2006).

Antifungal drug Amphotericin B (AmB) is used as alternative treatment in antimony resistant patients. Though highly effective against the disease, this drug has several limitations: (a) infusion related adverse reactions are common (b) it is much more expensive than sodium antimony gluconate (SAG), and (c) availability in India is quite erratic. Reports of unresponsiveness to Ambisome, a liposomal AmB formulation, in patients of VL is worrisome and indicates emergence of AmB resistant isolates (Sundar et al., 2002b; Muller et al., 2007). Miltefosine (MIL), an alkylphosphocholine, initially developed as an anticancer agent, showed selective activity against *Leishmania* (Croft et al., 2006a). Oral administration has the advantage of reducing socio-economic difficulties

that are present in areas of endemicity where health facilities are lacking. It was therefore proposed as the first line VL therapy and is the mainstay in the kala-azar (KA) elimination program in the Indian subcontinent (TDR, WHO, 2004).

Variation in the sensitivity to MIL has been observed in field isolates from Nepal & Peru with ED_{50} values ranging from less than 0.04 μg/mL to 8μg/mL (Yardley et al., 2005). The widespread use of MIL as a single agent in India and its long clinical half-life of approximately 7 days might lead to rapid emergence of resistance. Moreover, the ease with which resistant mutant parasites can be generated in the laboratory is a cause for concern (Seifert et al., 2003). Even before MIL is introduced into the market or into control programs, preliminary data from a phase IV trial in India involving domiciliary treatment and weekly supervision suggests doubling of the relapse rate (Bhattacharya et al., 2007). This provides a warning that Miltefosine resistance could develop quickly in field and plans are required to prevent it.

The development and spread of drug resistance has made surveillance of drug sensitivity a high priority issue. Earlier studies using isolates from antimony responsive and non responsive patients indicated that antimonial resistance is an intrinsic property of the parasite and is of acquired nature (Lira et al., 1999; Singh et al., 2006). Resistance development appears to be a feature of intensive transmission of *L. donovani* in Bihar.

The determinants for the inward translocation of MIL in *Leishmania* have been characterized in lab generated MIL resistant parasite as *L.donovani* putative Miltefosine transporter (LdMT; Perez-Victoria et al., 2003) and the putative β subunit for LdMT i.e LdRos3 (Perez-Victoria et al., 2006a). An impaired LdMT–LdRos3 dependent flippase

machinery at the plasma membrane was responsible for decreased drug uptake and therefore, a reduced susceptibility to MIL (Perez-Victoria 2003; 2006a; 2006b).

In the present study, *in vitro* antileishmanial activity of MIL in Indian field isolates of *L. donovani* was examined and compared with Amphotericin-B and SAG, two frequently used drugs for the treatment of VL. The correlation between MIL sensitivity and the mRNA expression of MIL transporters, LdMT and LdRos3(reported markers of MIL resistance), was also evaluated in Indian field isolates. In an effort to develop a high throughput drug screening assay, a fluorescence based resazurin assay was established as a simplified biological tool for drug susceptibility testing.

Patients, material and methods

Patients

Nineteen VL patients reporting to Safdarjung Hospital (SJH), New Delhi, were included in the study. The patients hailed from Bihar, India from zones of low and high endemicity for VL, which represent, respectively, the zones of low resistance (LR) and high resistance (HR) to antimony (Sundar, 2001). Three patients originated from neighbouring states of West Bengal or Uttar Pradesh. All patients received either SAG (20 mg /kg/ day intramuscularly for 30 days) or AmB treatment (infusions of 1 mg/kg on alternate days for 1 month). This work was conducted with the approval of the Ethics Committee of SJH and informed consent was obtained from all patients.

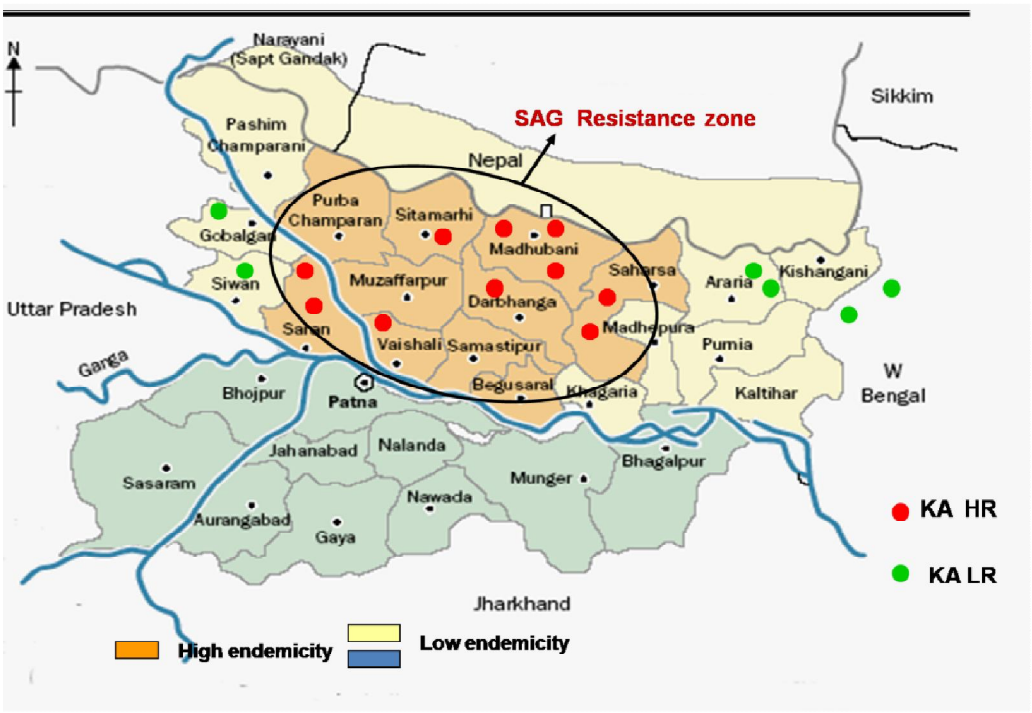


Figure 3.1: Map of Bihar and adjoining states showing origin of parasite isolates from areas of high and low endemicity to kala azar. Clinical isolates of *L. donovani* were prepared from patients originating from states of Bihar, West Bengal and Uttar Pradesh, India, hailing from zones of low and high endemicity for Visceral Leishmaniasis, which represent, respectively, zones of low resistance (KA LR, green dots) and high resistance (KA HR, red dots) to antimony.

Materials

Miltefosine was obtained from Cayman Chemical Company, USA, SAG (Albert David Ltd, India), AmB (Sigma, USA), Dulbecco`s modified eagle medium (DMEM: Sigma), Culture flasks (Corning, USA), Fetal Calf Serum (Biological Industries, Israel), 8-well Chamber slides (Nunc, USA), Diff-Quik stain solution (Dade Behring, USA), Trizol Reagent (Invitrogen, USA), DNAse I (Fermentas, USA), M-MLV Reverse transcriptase (Ambion, USA), oligo (dt) primer RETRO script (Ambion), SYBR Green

PCR Master mix (Applied Biosystems, USA), Resazurin (Sigma, USA) were employed for the study.

Leishmania donovani isolates

During 2001–2007, *L. donovani* isolates were prepared from bone-marrow aspirates of patients with KA (n=19). All the isolates were characterized as *L. donovani*, on the basis of species-specific PCR (Salotra et al., 2001). Parasites were routinely grown as promastigotes in Medium 199 (Sigma, USA) with 10% heat inactivated fetal calf serum (FCS, Biological Industries, Israel) at 26 °C. Antileishmanial drug susceptibility of all the isolates used in the study was determined in less than 6 passage after isolation from patients. Late log phase parasites, enriched with metacyclics, were used for assays to ensure that the parasites are closer to the situation in human. A standard strain of *L. donovani* (LdAG83) from India (MHOM/IN/83/AG83) was used as the reference parasite throughout the study.

Cell culture

Murine macrophage adherent cell line J774A.1 procured from NCCS, Pune, India, was maintained in DMEM medium supplemented with 1.5g/L sodium bi carbonate, 4.45 g/l glucose, penicillin (100 U/ml), streptomycin (100 mg/ ml) and 10% FCS at 37ºC in humidified atmosphere with 5% CO_2.

Susceptibility of promastigotes to antileishmanial drugs

L. donovani parasites in late log phase (1 x 10^6 cells/ml) were incubated with different concentrations of drugs (MIL - 0.25 to 15 µg/ml or AmB - 0.025 to 2µg/ml) for 72 hrs at 26ºC in 24 well tissue culture plate. The cell survival was measured by direct

counting under the microscope at 20X magnification. All the concentrations were tested in duplicates. Experiments were performed in triplicate. The percentage of killing was calculated and ED_{50} was determined using sigmoidal regression analysis (Origin 6.0; Origin Lab). The ED_{50} calculated by sigmoidal regression analysis were confirmed by repeating the drug assay at calculated ED_{50}.

Susceptibility of intracellular amastigotes to antileishmanial drugs

Murine macrophage adherent cell line J774A.1 in 8-well chamber slides (2 $X10^5$ cells/well) were infected with stationary-stage promastigotes at a 10:1 (parasite**:** macrophage) ratio and were incubated in 5% CO_2 for 4 h at 37ºC. Unattached promastigotes were washed off, and the cells were incubated for 18–24 h. Infected cells were re-incubated for 48 h, with different concentrations of drugs (SAG - 3, 10, 30, 60, and 100 µg/ml; MIL - 1, 2, 4 and 12 µg/ml; AmB- 0.025, 0.05, 0.1, 0.5, 1 and 2 µg/ml), while only DMEM with 10% FCS was added in control wells. Slides were stained with Diff-Quik solution and the number of *L. donovani* amastigotes was counted microscopically under oil immersion at X100 magnification in 100 macrophages. All the concentrations were tested in duplicate and the assay was performed in triplicate. The percent killing was calculated by sigmoidal regression analysis (Origin 6.0; Origin Lab), and the ED_{50} were determined. The ED_{50} calculated were revalidated by repeating the drug assay experiment with the ED_{50} concentration obtained by sigmoidal regression analysis.

RNA isolation, cDNA synthesis and Real-time quantitative PCR

Promastigotes were cultured and harvested at late log phase and immediately lysed in trizol reagent for RNA extraction following instructions recommended by the manufacturer. All samples were treated with DNAseI to remove possible contaminating genomic DNA. Total RNA (5µg/reaction) was reverse transcribed at 42ºC with M-MLV reverse transcriptase using conditions recommended by the manufacturer with a 18mer oligo (dt) primer RETRO script. The resulting cDNA was diluted 10 times, and 2 µl was added to 25 µl quantitative PCR reactions for gene expression profiling of LdMT and LdRos3, referred to as target genes, and of GAPDH gene, included for normalization purpose, referred to as internal control. The sequences of the primers for all the genes amplified in the study are given in the Table 3.1.

Table 3.1: Oligonucleotide sequences of genes amplified by Real time PCR

Gene	**Forward Primer** **Primer Sequence (5'→ 3)**	**Reverse Primer** **Primer Sequence (5'→ 3)**	**Product size** **(bp)**
GAPDH	GAA GTA CAC GGT GGA GC TG	CGC TGA TCA CGA CCT TCT TC	206
LdMT	CAA GTG CCT TTC CAC AG AAT C	CTC ACC TTT TTG AAC TCC AAC AGG	228
LdRos3	ACG ACA CGG CTT GAT TT CG	GAG TAG TCC ACG GAG GCA GTA AAG	238

Foot note: GAPDH: glyceraldehyde-3-phosphate dehydrogenase, LdMT: *L.donovani* putative Miltefosine Transporter, LdRos3: ß-subunit of LdMT

All Real time PCR reactions were performed in triplicate using SYBR Green PCR master mix for detection in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). A singleplex reaction mix was prepared according to the manufacturer's

protocol. The reactions were run using the thermal profile consisting of three stages: (1) incubation at 50ºC for 2 min, (2) incubation at 95ºC for 10 min, and (3) incubation at 95ºC for 15 sec, then at 60ºC for 1 min. The third stage was repeated for 40 cycles. A dissociation protocol was performed with 60°C set as the starting temperature with increments of 0.5°C every 30 s to ascertain amplification of the expected product and to ensure no nonspecific products or primer dimers (which could bias the quantification) were formed. The negative controls of cDNA synthesis (i.e., without reverse transcriptase), and no-template controls were included in each run for each gene. All reactions were performed in triplicate and the arithmetic average threshold cycle (Ct) was used for data analysis.

PCR efficiency was measured using a standard curve generated by serial dilutions of RNA, starting with the initial RNA concentration of 100 ng/µl which was serially diluted 10 fold (100ng, 10ng, 1ng, 0.1ng, and 0.01 ng) for the real time PCR assay for generating relative standard curve for quantitation (ABI ,1997). The PCR efficiency (E) was calculated by the formula: $E=10^{(1/\text{-slope})}-1$, and ranged from 90-100% in the different assays (Fig 3.2).

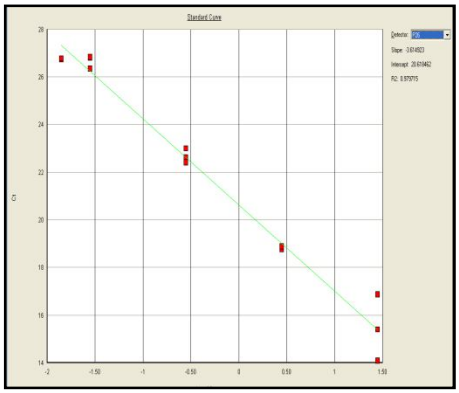


Fig 3.2: Standard curve for determining the primer efficiency of GAPDH obtained by Real time PCR. The cDNA concentration is plotted against Ct values.

Analysis of gene expression using $2^{-\Delta\Delta Ct}$ method.

We used the $2^{-\Delta\Delta Ct}$ method to calculate relative changes in gene expression determined from real time quantitative PCR experiments. The data are presented as the fold change in the target gene expression in the *L. donovani* field isolates normalized to the internal control gene (GAPDH) and relative to the reference isolate of *L. donovani* *Ld*AG83 or *Ld*M2*0*. The average Ct was calculated for both the target genes and GAPDH and the ΔCt was determined as (Ct values for the target gene) minus (Ct values for GAPDH). The -ΔΔCt represents the difference between the paired isolate samples, as calculated by the formula -ΔΔCt = (ΔCt of field isolate - ΔCt of *Ld*AG83 or *Ld*M2*0*). The N-fold differential expression in the target gene of a field isolate compared to the *Ld*AG83 was expressed as $2^{-\Delta\Delta Ct}$. In the present study, mRNA expression profile categorized as follows: N fold ≥ 1.5, designated as increased, N-fold ranging from 1to 1.49 as normal, and N-fold ≤ 0.75 as decreased.

Establishment of fluorescence bioassay for high throughput drug screening in *L.donovani*

In an effort to develop a high throughput drug screening method, a fluorimetric bioassay was standardized in the lab to establish the relative cytotoxicity of antileishmanial agents simultaneously in a large number of parasite samples at promastigote stage.

Preparation of resazurin stock

Resazurin stock was prepared by mixing equal quantities of potassium ferricyanide and potassium ferrocyanide (30mM each) with 3mg/ml resazurin prepared in 0.1M potassium phosphate buffer (PPB), pH 7.4 and diluting it to 1:20 in PPB.

***In vitro* resazurin based assay for determination of drug sensitivity of *L.donovani* promastigotes**

Promastigotes were seeded into the wells at 10^5 promastigotes/well and exposed to the 2 fold serially diluted MIL concentrations (0.16 to 160 µg/ml) in 96-well plate and incubated for 72h at 26°C. After the stated incubation period, 50µl of resazurin stock was added to each well and the plates are further incubated for 24h at 26°C. Fluorescence measurement was taken using the filter combination 550nm exitation-590nm emission (Infinite M200, Tecan, Switzerland). ED_{50} was determined using Microcal Origin 6.0 software as described above.

Statistical analysis

All experiments were conducted at least in triplicate and results are expressed as mean ± SEM. Student's unpaired *t* test was used to determine the statistical significance of the values obtained. The Mann-Whitney U test was used to determine the statistical significance of the mean ED_{50} values obtained (GraphPad Prism software Ver. 5.0, GraphPad software Inc, California). Karl Pearson's correlation coefficient was calculated to determine the correlation between the *in vitro* sensitivities of promastigote and amastigotes. Spearman's rank correlation coefficient was calculated to determine the

correlation between the endemicity zones, *in vitro* susceptibility, and the clinical response of patients. Statistical significance was accepted as $p<0.05$.

Results

Susceptibility of *L. donovani* isolates to antileishmanial drugs

Susceptibility profile of VL field isolates (n=19) obtained from patients hailing from low and high VL endemicity regions was determined for SAG, AmB two widely used drugs for treatment and for MIL, a newly introduced oral drug. The clinical profile of VL patients and *in vitro* susceptibility of VL isolates to SAG, MIL and AmB is summarized in Table-3.2.

In vitro susceptibility to Amphotericin B at promastigote and intracellular amastigote stage

The field isolates exhibited variable susceptibility to AmB in *in vitro* assays at intracellular amastigote and in promastigote stages. The ED_{50} for AmB determined at promastigote stage ranged from 0.312 ± 0.014 to 1.62 ± 0.134 mg/ml with mean ED_{50} as 0.89± 0.44 mg/ml. The ED_{90} values for AmB ranged from 0.37±0.02 to 2.55±0.42 mg/ml with mean ED_{90} of 1.29±0.63µg/ml. The ED_{50} (± SEM) value of AmB at amastigote stage ranged from 0.17±0.01 to 0.77± 0.08 µg/ml with mean ED_{50} (± SEM) of 0.39±0.19 µg/ml (Fig 3.3, Table 3.2).

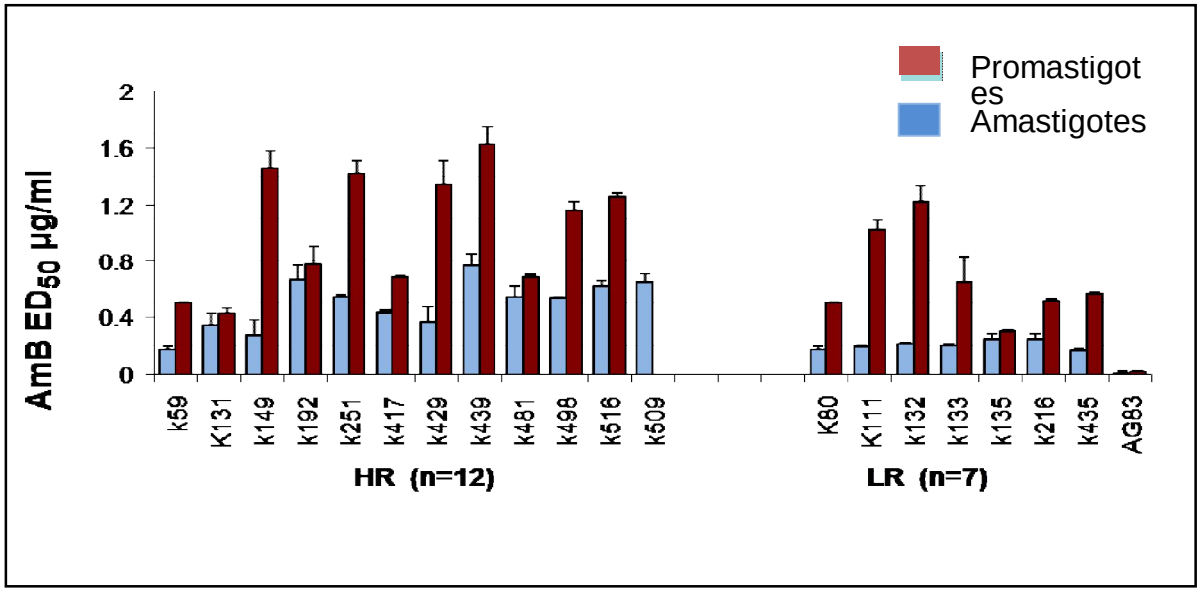


Figure 3.3: ***In vitro*** **susceptibility of** ***L. donovani*** **field isolates towards Amphotericin B**. The *in vitro* AmphotericinB susceptibility of VL isolates was determined in SAG resistant (HR) and SAG sensitive (LR) field isolates at both promastigote and intracellular amastigote stages. AG83 was used as reference *L.donovani* parasite.

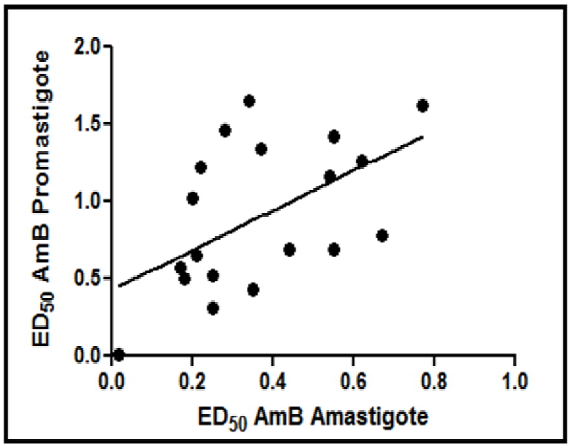
Antileishmanial activity of AmB correlated partially (r=0.58) at amastigote and promastigote stages (fig 3.4). Promastigote stage of field isolates exhibited generally higher or equal ED_{50} in comparison to amastigote stage.

10 of the 19 patients were treated with AmB and all responded well to treatment. Isolate derived from AmB responsive patient of the LR zone displayed the highest susceptibility in *in vitro* assay (Table 3.2). The WHO reference isolate *Ld*AG83 was the most susceptible isolate (~ 10 times more in comparison to the most susceptible field isolate) at both promastigote and amastigote stage.

TABLE 3.2. Clinical profiles of VL patients from LR and HR regions and in vitro susceptibility of parasite isolates to SAG,

ID	Sex/ Age	Area/Endemicity Region[a]	Treatment/ Response[b]	SAG[c] (Amastigotes) ED_{50} (µg/ml)	AmB[c] (Amastigotes) ED_{50} (µg/ml)	AmB[c] (Promastigote) ED_{50} (µg/ml)	MIL[c] (Amastigote) ED_{50} (µg/ml)	MIL[c] (Promastigote) ED_{50} (µg/ml)
K59	F/21	Vaishali, HR	SAG (NR)[d]	14.66 ± 3.29	0.180 ±0.02	0.50 ± 0.006	1.86 ± 0.07	1.19 ± 0.07
K131	M/22	Saharsha, HR	SAG (NR)	19.38 ± 1.68	0.35 ± 0.08	0.43 ± 0.035	0.51 ± 0.06	0.63 ± 0.03
K149	M/20	Saran, HR	AMB (R)	15.70 ± 4.01	0.28 ± 0.10	1.46 ± 0.12	1.57 ± 0.16	1.56 ± 0.11
K192	M/24	Saran, HR	Amp-B (R)	20.30 ± 0.84	0.67 ± 0.10	0.78 ± 0.12	1.99 ± 0.17	1.91 ± 0.08
K251	M/11	Saran, HR	ND[f]	11.82 ± 1.28	0.55 ± 0.01	1.42 ± 0.09	1.59 ± 0.07	0.40 ± 0.05
K417	F/8	Muzaffarpur,HR	Amp-B (R)	14.65 ± 0.67	0.44 ± 0.01	0.69 ± 0.007	1.90± 0.04	1.72 ± 0.11
K429	M/26	Saharsha, HR	Amp-B (R)	13.76 ± 0.82	0.37± 0.10	1.34 ± 0.17	1.24 ± 0.18	1.26± 0.06
K439	M/16	Muzaffarpur,HR	Amp-B (R)	12.88 ± 0.12	0.77 ± 0.08	1.62± 0.134	1.07 ± 0.1	0.87 ± 0.04
K481	M/32	Muzaffarpur,HR	Amp-B (R)	17.53 ± 0.34	0.55 ± 0.07	0.69±0.016	2.32 ± 0.14	1.90 ± 0.12
K498	F/55	Madhubani, HR	Amp-B (R)	15.82 ± 0.24	0.54± 0.01	1.16 ±0.06	1.65 ± 0.05	1.32 ± 0.08
K516	F/60	Motihari, HR	Amp-B (R)	16.48 ± 0.61	0.62 ± 0.04	1.26±0.029	2.02 ± 0.10	1.62 ± 0.16
K509	F/4	Madhubani ,HR	Amp-B (R)	16.84 ± 0.26	0.65 ± 0.06	ND	2.16 ± 0.13	1.94 ± 0.11
K80	F/40	Bhagalpur, LR	SAG/ (NR)	10.42 ± 2.17	0.18 ± 0.02	0.50 ±0.006	1.32 ± 0.04	1.58 ± 0.16
K111	F/36	Siwan , LR	SAG (R)	5.63 ± 0.57	0.20 ± 0.01	1.02 ± 0.07	0.85 ± 0.19	0.47 ± 0.06
K132	F/24	Munger, LR	ND[f]	3.95 ± 0.28	0.22 ± 0.01	1.22 ± 0.11	0.48 ± 0.05	0.53± 0.03
K133	M/20	West Bengal,LR	SAG (R)	3.45 ± 0.28	0.21± 0.01	0.65 ± 0.18	0.93 ± 0.10	0.83 ± 0.04
K135	F/45	Gopalganj, LR	SAG (R)	4.22 ± 0.38	0.25 ± 0.04	0.31 ± 0.014	0.72 ± 0.03	0.86 ± 0.03
K216	M/14	West Bengal.LR	SAG (R)	2.14 ± 0.28	0.25 ± 0.03	0.52 ± 0.018	0.91± 0.19	0.76 ± 0.02
K435	M/17	Kushinagar, LR	Amp-B (R)	11.82 ± 1.39	0.17 ± 0.01	0.57 ± 0.012	1.08 ± 0.25	1.16 ± 0.19
LdAG83		Ref L. *donovani*	NA[g]	2.06 ± 0.23	0.017 ± 0.01	0.023± 0.01	0.85 ± 0.03	1.05 ± 0.07

M, male; F, female. HR- high endemicity area, LR-Low endemicity area *b* Responses were noted 30 days after treatment with SAG infusions (20 mg/kg of body weight) or with AmB infusions (1 mg/kg of body weight) on alternate days for 1 month. Patients with an absence of fever and with a reduction in spleen size were designated responders (R); patients who did not exhibit those outcomes were considered nonresponders (NR).*c* Mean ED50s±SD (µg/ml) of the results from 3 separate assays..*f* ND, not determined .*g* NA, not applicable.



Number of XY Pairs	20
Spearman r	0.5782
95% confidence interval	0.1685 to 0.8175
P value (two-tailed)	0.0076
P value summary	**
Exact or approximate P value?	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	Yes

F**igure 3.4: Correlation of AmB susceptibility at promastigote and intracellular amastigote stage.** The *in vitro* susceptibilities of promastigote and amastigote parasites stages towards AmB correlated partially (r=0.58). This includes the susceptibility of standard LdAG83 parasite isolate.

In vitro susceptibility to Miltefosine (MIL) at promastigote and amastigote stages

The ED_{50} (± SEM) for MIL against promastigotes ranged from 0.40 ± 0.05 to 1.94± 0.11 mg/ml with mean ED_{50} as 1.18 ± 0.11 mg/ml whereas ED_{90} ranged from 1.64 ± 0.34 to 8.83 ± 2.67mg/ml with mean ED_{90} of 3.98 ± 0.87mg/ml (Fig 3.5, Table 3.2). In an intracellular amastigote macrophage assay, the field isolates showed variable susceptibility to MIL with ED_{50} ranging from 0.48 ± 0.05 to 2.32 ± 0.14 mg/ml and the ED_{90} from 3.53± 0.12 to 12.83±1.2 mg/ml. The mean ED_{50} for field isolates as amastigote was 1.38 ± 0.55 mg/ml, while mean ED_{90} was 6.59 ± 3.22 mg/ml.

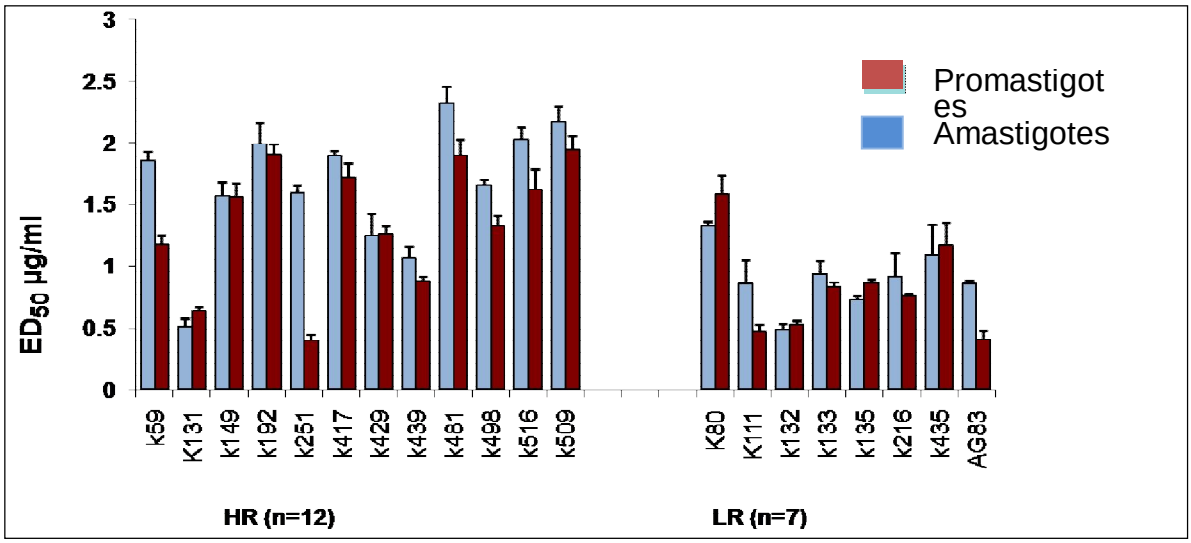
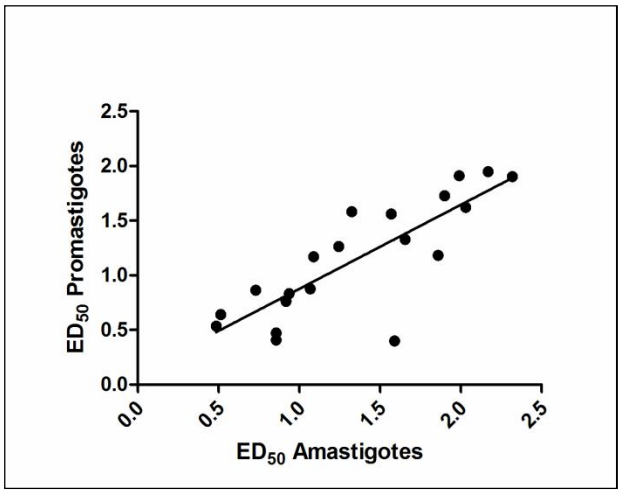


Figure 3.5*: In vitro* susceptibility of field isolates towards Miltefosine. The *in vitro* MIL susceptibility of VL isolates was determined in SAG resistant (HR) and SAG sensitive (LR) parasites at both promastigote and intracellular amastigote stages. AG83 was used as reference *L. donovani* parasite.

Previous studies indicated that susceptibility to MIL at both the stages correlate fairly well (Escobar et al., 2002). Here, evaluation of the antileishmanial activity of MIL at the two stages of parasite in 19 field isolates revealed a strong correlation between the susceptibility of amastigotes and promastigotes (r_{rank} =0.81) (Fig 3.6).



Number of XY Pairs	20
Spearman r	0.8101
95% confidence interval	0.5633 to 0.9242
P value (two-tailed)	P<0.0001
P value summary	***
Exact or approximate P value?	Exact
Is the correlation significant? (alpha=0.05)	Yes

Figure 3.6: Correlation of MIL susceptibility at promastigote and intracellular amastigote stage. The *in vitro* susceptibilities of promastigote and amastigote parasites stages towards MIL correlated significantly (r=0.81).

Comparison of ED_{50} values of isolates from different endemicity zones and Correlation with SAG susceptibility

The mean ED_{50} values at the clinically relevant stage i.e. intracellular amastigote were compared in isolates originating from high and low endemicity zones as well as SAG resistance zone (Sundar, 2001). The mean and median ED_{50} of SAG in isolates from HR region (15.81 ± 2.50 and 15.76 mg/ml, respectively) was significantly higher ($p<0.001$) in comparison to the ED_{50} of LR (5.46 ±3.69 and 4.22 mg/ml respectively) region isolates (Fig 3.7A). We observed strong correlation of *in vitro*

susceptibility with the endemicity zones of the isolates and with the clinical response for SAG.

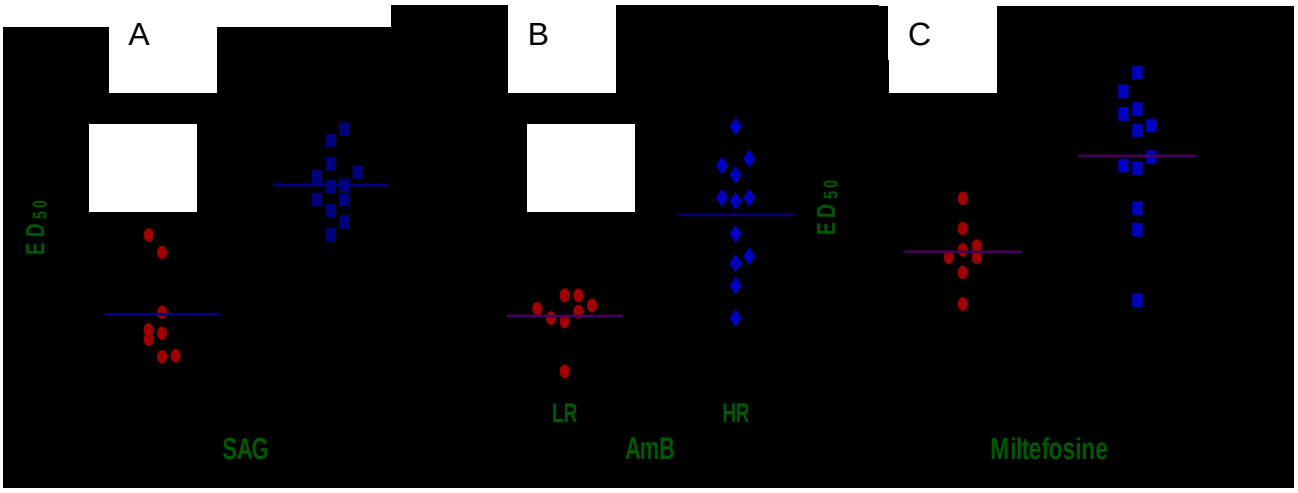


Figure 3.7: Susceptibility of *L .donovani* parasites from different endemicity zones to SAG, Miltefosine and Amphotericin B. The mean ED_{50} of isolates from HR region was significantly higher in comparison to the ED_{50} of LR region isolates for all three drugs (A) SAG, (B) AmB and (C) MIL. ED_{50}± SEM represented for all drugs is in mg/ml.

In case of AmB, the mean ED_{50}± SEM (0.49 ± 0.17 mg/ml) of isolates from HR region were found to be significantly higher ($p<0.001$) in comparison to the mean ED_{50} (0.21 ± 0.03mg/ml) for LR zone isolates (Fig 3.7 B). Similarly, in case of MIL, the isolates from HR zones displayed the mean ED_{50} as 1.65 ± 0.14 mg/ml and median ED_{50} as 1.75 mg/ml, which was significantly higher ($p<0.01$) than the isolates from LR region where the corresponding value was 0.90 ± 0.1mg/ml and 0.91mg/ml ($p<0.001$), respectively (Fig 3.7 C). Overall, we observed a significantly positive correlation between the *in vitro* SAG susceptibility profile with AmB ($r= 0.599$, $p<0.01$) or MIL ($r = 0.66$, $p<0.01$). MIL and AmB sensitivity profiles for *Leishmania* field isolates were positively correlated ($r = 0.57$, $p<0.01$) (Fig 3.8).

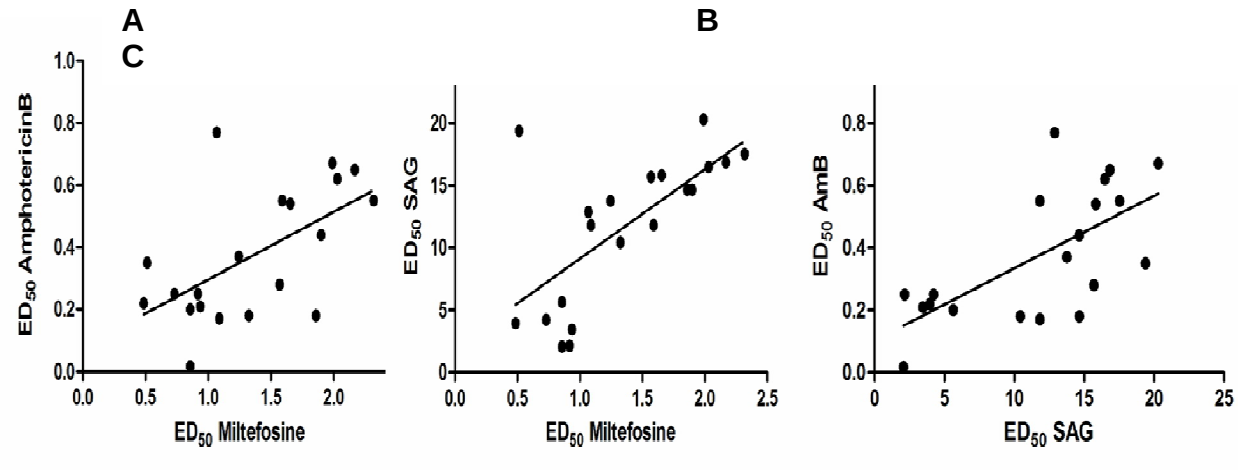


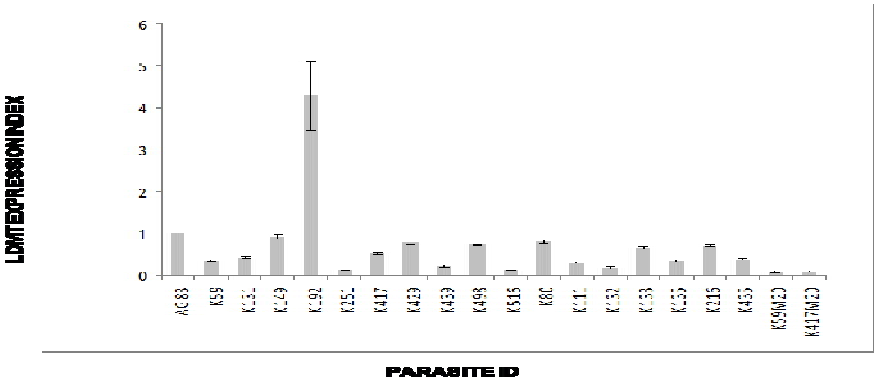
Figure 3.8: Comparison of the *in vitro* drug susceptibility profiles of various antileishmanial drugs in *L. donovani* isolates. A significantly positive correlation was observed between the susceptibility profile of (A) MIL with AmB (r = 0.57, p<0.01) (B) MIL with SAG (r = 0.66, p<0.01) or (C) SAG with AmB (r= 0.599, p<0.01).

Expression of LdMT and LdRos3 in *L.donovani* isolates

MIL uptake is reported to correlate well with the sensitivity to the drug. The uptake levels and therefore the sensitivity depend on the expression levels of the functional flippase machinery at the plasma membrane constituted by transporter LdMT and its subunit LdRos3 (Perez Victoria et al., 2003; 2006a). The gene expression was evaluated in VL field isolates (n=17) for LdMT and LdRos3 in comparison with the expression in *Ld*AG83 standard *L. donovani* strain and MIL resistant parasite *Ld*M20, adapted to 20mg/ml MIL (Table 3.2). In comparison to *Ld*AG83, majority of the isolates revealed down regulated expression of LdMT and LdRos3 (Fig 3.9). In general, the ratio of expression of LdMT and LdRos3 varied greatly among isolates (from 0.67 to 10 fold).

In comparison to lab generated MIL resistant parasite LdM20 the expression of LdMT and LdRos 3 was more than 3 fold higher in majority of the isolates (14/17, 82.3%) which is suggestive of an alternate mechanism operating in the field towards MIL activity.

A



B

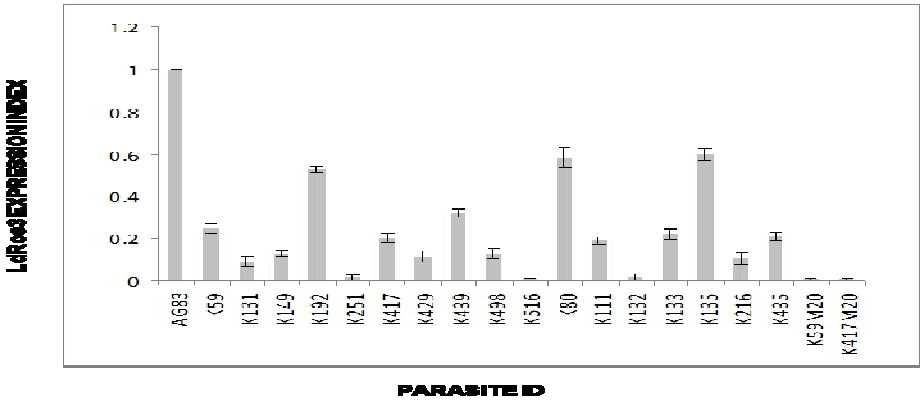


Figure 3.9: Expression of LdMT and LdRoS3 in field isolates. (A) Real time RT-PCR expression analysis of *L. donovani* Miltefosine transporter (LdMT): Graph shows expression index defined as ratio of the expression of LdMT in *L.donovani* field isolates relative to the LdAG83. (B) Real time RT-PCR expression analysis of LdRos3 as expression index relative to LdAG83. MIL resistant parasites, K59M20 and K417M20 (described in chapter 5) were used as positive control in the study. The values expressed are Mean ± SD of three independent experiments performed in triplicate.

Establishment of Resazurin assay as a simplified biological tool for high throughput drug screening

There is a strong correlation between Miltefosine (MIL) susceptibility at promastigote and amastigote parasite stages and therefore resazurin based promastigote susceptibility assay can be directly applied as a simplified biological tool to determine the MIL sensitivity in field. The resazurin assay is based on non fluorescent redox indicator, resazurin which gets converted to fluorescent reporter molecule resorufin by the reducing capability of metabolically active cells (Tote et al., 2009). The fluorescence intensity is the function of cell viability. The assay is now well standardized in our lab for high throughput screening of antileishmanial drugs (Fig 3.10).

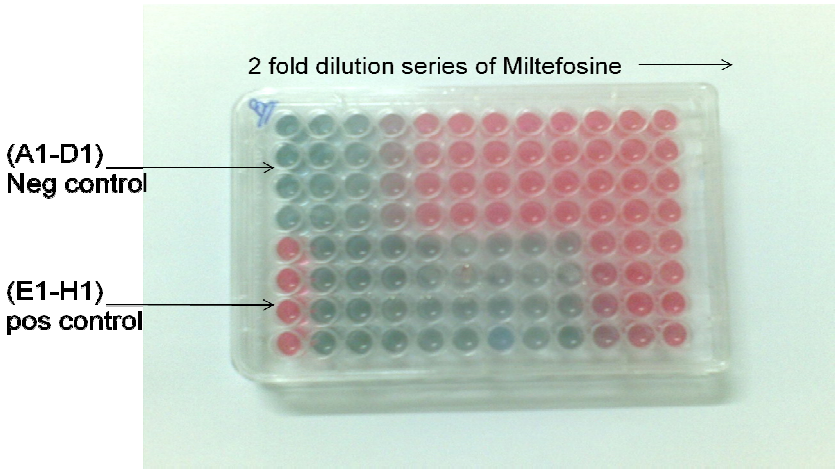


Figure 3.10: Resazurin based assay as a simplified biological tool for promastigote viability testing: Neg Control: Wells containing only medium, Pos control: Parasites in medium, no drug added. Depending on the number of viable cells, blue coloured, non fluorescent resazurin gets converted to pink coloured resofurin. The fluorescent signal is measured with 550nm excitation and 590 nm emission filter.

Discussion

Leishmaniasis is considered to be the second most important protozoal disease and one of the neglected diseases that has become a special focus for WHO. Resistance to antimonials has emerged as a major hurdle to the treatment and control of VL in India. At present, the disease is treated with pentavalent antimony as well as with the antifungal drug Amphotericin-B. Miltefosine (MIL), recently introduced for treatment in the VL control programme, is a major breakthrough in the therapy; but oral bioavailability is the paradoxical blessing as MIL can be used widely on an outpatient basis thus improving coverage; however, this also exposes the drug to misuse. Unsupervised use of MIL will have heavy consequences in terms of both its safety and useful therapeutic lifespan. The present study considered 19 *L. donovani* isolates from Indian patients of KA who were from zones of varying degree of endemicity. *In vitro* susceptibility of parasites isolated from SAG treated (responsive and non responsive) and AmB treated patients (all responded to treatment and no clinical resistance was observed) was studied. The field isolates of *L. donovani* were investigated for susceptibility towards SAG, AmB and MIL in *in vitro* assay at intracellular amastigote stage and promastigote stage.

Results indicated that the isolates from HR region had higher tolerance towards all three drugs studied compared to LR region isolates. *In vitro* SAG refractoriness in HR zone isolates has been shown earlier (Sundar, 2001; Singh et al., 2006) where the patients are largely unresponsive to SAG. *In vitro* susceptibility of isolates to all the three evaluated drugs significantly correlated with one another, raising possibility of mechanism of cross- resistance operating in field isolates. Here, we observed varied ED_{50} of field isolates and all 10 of the patients treated with AmB responded to the therapy.

Although we have not observed any clinical AmB resistance, a significant higher ED_{50} in isolates from high endemicity zone indicates the possibility of emergence of resistance as observed in Sudan (Mueller et al., 2007). MIL cure rates might vary in different geographical areas depending on the prevalent species. Miltefosine has shown a 94% cure rate of VL patients with a dose of 2.5 mg/kg daily for 28 days in Phase IV trial in India (Sundar et al., 2002a) while its efficacy varied against American CL caused by *L. panamensis* and *L. braziliensis*, with a respective cure rate of 66% and 94% (Soto et al., 2001). In a recent study, a significant variation in MIL sensitivity has been demonstrated in clinical isolates of different *Leishmania* spp. from Peru and Nepal (Yardley et al., 2005).

We observed the variable susceptibility of field isolates in case of MIL that correlated well with the SAG resistance and high endemicity zone indicating that in India, unresponsiveness to MIL may emerge earlier in high endemicity zone. Long-terminal half-life of 7 days might encourage the emergence of resistance, as sub-therapeutic levels of MIL would remain for weeks after a standard course of treatment (Bryceson, 2001). *Leishmania* parasite resistant to various drugs have been generated by lending continuous exposure to drug in laboratory for SAG, AmB and MIL and have been used as model organism to understand the resistance phenomenon in various *in vitro* studies (Mbongo et al., 1998; Seifert et al., 2003). In *in vivo* studies, susceptibility of *L. donovani* has been shown to decrease after one or more course of treatment either with SAG or AmB (Faraut-Gambarelli et al., 1997; Di Giorgio et al., 1999). Clinical resistance has been indicated in Indian VL patients since relapses were observed in Phase IV MIL trials (Bhattacharya et al., 2007). Considering that multidrug resistance is a manifestation

of multifactorial phenomena, various determinants may be responsible for variation in drug susceptibility of field isolates and possibility of cross-resistance cannot be ignored. The possible explanation for the differences in the ED_{50} between high and low endemicity regions for the three drugs can be attributed to differences in membrane sterol (Beach et al., 1988) and lipid content (Beach et al., 1979), have been demonstrated to have distinct drug susceptibility profile. The extensive use of SAG in these areas may have changed the biochemical composition of these parasites' membrane which might affect drug susceptibility. Both AmB and MIL are known to interact with the plasma membrane of the cells and membrane modifications are also suggested as a mechanism of resistance in SAG resistant isolate (Rakotomanga et al., 2004). Several studies confirmed that AmB binds with the sterols, preferentially with ergosterol in cell membrane and causes the formation of pores in the membrane that alters the permeability to ions leading to cell death (Brajtburg & Bolard, 1996; Azas et al., 2001). C-24-alkylated sterols selectively interacting with AmB are absent in *L. donovani* promastigotes resistant to AmB (Pourshafie et al., 2004). 24-alkylated sterols content was found halved in MIL resistant parasites than wild-type promastigotes at the plasma membrane level (Rakotomanga et al., 2005) indicating that membrane modification may be responsible for variable susceptibility. It is evident from various reports that SAG as well as MIL and AmB kill cells by a process with several features characteristic of programmed cell death (Sereno et al., 2001; Lee at al, 2002; Verma & Dey, 2004). An Indian antimony resistant field isolate has been shown to exhibit cross-resistance to MIL and AmB. Characterization of the same isolate in a proteomic study revealed that HSP83 and a small kinetoplastid calpain-related protein play a key role in antimonial resistance by

modulating drug-induced programmed cell death (Vergnes et al., 2007). These studies indicate that there may be some common mechanism of resistance, like permanent modification in the membranes or drug transporters etc. that may modulate drug induced cell death is operating in isolates and may lend cross resistance to the drugs.

The current study suggests a strong correlation between the activity of MIL at promastigote and amastigote stages in field isolates indicating the utility of the easier *in vitro* drug susceptibility assay based on the promastigote form for monitoring MIL susceptibility in endemic area. We observed a moderate correlation for AmB activity at two stages and amastigotes were more susceptible in comparison to promastigote. The increased susceptibility of isolates at intracellular stage may be attributed to the fact that activity of AmB appears to be dependent on macrophage interactions as it enhances nitric oxide synthesis by murine macrophage J774.A.1 (Mozaffarian et al., 1997). Towards the development of a simplified biological tool for high throughput screening of MIL at promastigote stage in field, fluorescence based resazurin assay was established (Tote et al., 2009). The assay has immense advantage over the direct counting method as it is accurate, rapid, and convenient and can analyze a large number of samples. The efficacy of the method is comparable to highly sensitive Alamar blue assay; however, the method is much more economic and hence can prove to be very useful field based assay in resource poor endemic areas of VL in India.

It was proven in the laboratory generated MIL resistant isolate that, MIL sensitivity correlates with the level of drug uptake, which depends on the expression level of the two transporters LdMT and LdRos3. The field isolates were all found susceptible

to MIL and the expression of these markers of resistance were not significantly down regulated. Further, no correlation was observed between the mRNA expression of LdMT and LdRos3 and *in vitro* MIL susceptibility of field isolates, though the experimental MIL resistant parasites evaluated as controls displayed significantly down regulated expression of these two genes (discussed in detail in chapter 5). When the expression profile was compared with *Ld*AG83, a reference isolate, all field isolates except one displayed comparable expression of both the molecules, indicating that other yet to be discovered mechanism may be operative in the field isolates. Therefore, LdMT/LdRos3, implicated in experimental resistance to MIL, did not appear to be suitable markers to monitor drug susceptibility in field isolates.

The present study highlights the incidence of higher tolerance to the three drugs i.e. SAG, AmB, and MIL in field isolates from high endemicity zone as they invariably displayed decreased susceptibility towards these drugs emphasizing the need for novel treatment strategies against VL. Emergence of antimony resistance in anthroponotic VL cycle suggests that resistance could also develop to other antileishmanial drugs once they are widely used as single agent. Studies to test the combination treatment comprising MIL or liposomal amphotericin B along with a drug that has different mechanism of action like paromomycin (recently granted marketing authorization in India) have been initiated to make the treatment of VL more effective, attractive, and available to all sections of the society. This will help to improve compliance and prolong the useful therapeutic life span of the drugs.

In vitro assessment of sensitivity of Kala azar isolates towards new/ potential antileishmanial drugs: Paromomycin, Sitamaquine and Marine extracts

Introduction

The present treatment of visceral leishmaniasis (VL)/ kala azar (KA) is unsatisfactory in India and is therefore undergoing transition in terms of choice of the first line treatment since more than half of the VL patients in India, are resistant to traditional SAG therapy (Sundar et al., 2000; Das et al., 2005). Down slope of SAG efficacy has led to the use of alternative anti-leishmanials such as amphotericin B and miltefosine. These drugs, although highly effective, are faced with problems of toxicity, high cost and risk of emergence of resistance particularly to miltefosine which has a long half life (Sundar et al., 2002; Boer et al., 2006; Perez-Victoria, 2006a).

The arsenal of antileishmanial drugs is limited and only few drugs are under development. Paromomycin (PMM) and sitamaquine (SIT) are the new drugs with promising antileishmanial efficacy. PMM is an aminoglycoside antibiotic showing efficacy against VL (Jha et al., 1998; Thakur et al., 2000; Croft & Yardley, 2002), with overall cure rates above 90%. As a result, it was registered in India in August 2006 and may prove a promising public health tool in the nationwide VL elimination programme (Sundar & Chakravarty, 2008). It has successfully completed phase IV clinical trials, showing advantages of shorter treatment course, higher safety profile and low manufacturing cost, making it an affordable therapeutic option in Indian field conditions (Sundar et al., 2007; Sinha et al., 2011). SIT, an 8 aminoquinoline, is an oral antileishmanial drug that has completed phase IIa clinical trials with more than 85% cure rates and is currently under phase II b clinical trials (Sherwood et al., 1994; Jha et al., 2005). The major limitation regarding SIT is the unavailability of sufficient data on the pharmacokinetics of the drug and its metabolites and toxicity concerns such as renal

adverse events and methemoglobinemia in treated kala azar patients. Pharmacokinetic analysis of SIT in dogs indicated that it has relatively high systemic clearance, large volume of distribution, relatively short half-life and low systemic availability (Taylor et al., 1991).

With very few chemotherapeutic alternatives in hand, the primary concern should be to protect the available drugs against development of resistance by formulating and implementing the right policies for administering these drugs. *In vitro* SAG efficacy is reported to correlate with the clinical outcome (Lira et al., 1999; Singh et al., 2006). Current first line treatment choices for VL are miltefosine and amphotericin B. However, SAG resistant isolates exhibit a higher tolerance towards both these drugs (Kumar et al., 2009b). The future of VL chemotherapy now banks on combinatorial treatment that could improve the efficacy by reducing the duration or total drug dosage (and therefore delay resistance), lower the toxicity and cost leading to higher patient compliance. Combination therapy using amphotericin B or its liposomal formulation along with miltefosine or PMM has shown promising results (van Griensven et al., 2010). Determination of *in vitro* susceptibility of natural *Leishmania* parasite population towards new treatment options will help to develop effective control measures. In the present work, we have attempted to investigate the susceptibility of *L. donovani* isolates derived from Indian VL patients hailing from zones of varying degree of SAG resistance towards PMM and SIT. Further, in order to understand how these drugs mount their cytotoxic effect on the intracellular parasites, we evaluated the effect of PMM/SIT treatment on the release of nitric oxide (NO) by *L. donovani* infected macrophages (Bogdan et al., 2000).

With the recent emphasis of the WHO on the development of antileishmanial agents from natural products, an urgent search for drugs derived from terrestrial plants and marine flora/ fauna has been initiated. Many natural products have been shown to have antiprotozoal properties *in vitro* (Dube et al., 2007; Freile-Pelegrin et al., 2008). Secondary metabolites produced in marine organisms could be the source of bioactive substance and useful in modeling compounds for drugs (Faulkner, 2001; Haefner, 2003). Marine sponges are shown to exhibit antibacterial, insecticidal, antiviral, and antiplasmodial activities (Rao et al., 2003; Yan et al., 2004). In the frame of searching for new leads against VL cure, we evaluated the antileishmanial potential of 25 methanolic extracts obtained from different species of marine organisms (sponges, mollusks, coelenterates, ascidians) in standard *L.donovani* parasite (LdAG83).

Patients, materials and methods

Patients

To study drug responses, clinical isolates of *L. donovani* (n=20) were prepared as described in Chapter 3, with the addition of two new patients reporting to SJH in 2008 (Fig. 4.1, Table 4.1).

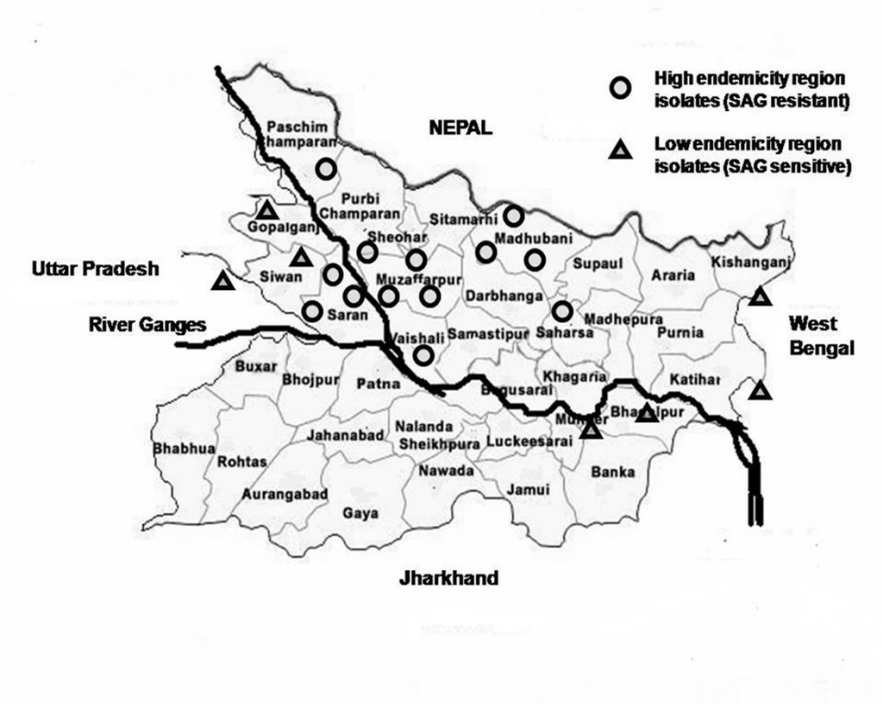


Figure 4.1: Map of Bihar and adjoining states showing origin of parasite isolates from areas of high and low endemicity. Clinical isolates of *L. donovani* were prepared from patients originating from states of Bihar, West Bengal and Uttar Pradesh, India, hailing from zones of low and high endemicity for visceral leishmaniasis, which represent, respectively, zones of low resistance (LR) and high resistance (HR) to antimony. Modified from the district map of Bihar available at http://www.biharonline.gov.in.

Materials

The antileishmanial drugs viz. paromomycin sulfate (Sigma Aldrich, USA), sitamaquine (Glaxo Smith Kline, UK) and SAG (Albert David, India) were employed in this study. Stock solutions for PMM and SIT (10mM each) were prepared freshly in deionized water and filter sterilized. 1 mM N-methyl-L-arginine monoacetate (L-NMMA) (Calbiochem, USA), lipopolysaccharide (LPS) from *E.coli* (LPS: Sigma, USA), Griess reagent (Sigma, USA), sodium nitrite (Sigma, USA) were employed in the study. Other cell culture reagents were used as described in chapter 3.

Preparation of dilutions of crude marine extracts

25 methanolic extracts of marine organisms collected from Rameshwaram, India (90 15'N; 790 15'E) were provided by Dr K. Padmakumar, Department of Aquatic Biology & Fisheries, University of Kerala, Thiruvananthapuram, India. The crude, dried, methanolic extracts were stored at -20°C until use. These extracts were dissolved freshly in water and filter sterilized (0.22 μm membrane filter) to prepare stock solutions. These stock solutions were further diluted in the appropriate medium (M199 or RPMI supplemented with 10% FCS) and used immediately for the drug susceptibility assays.

Drug susceptibility assays

The stationary phase promastigotes were subjected to drug screening assay by plating at $1X10^6$ parasites/well in 24 well plates containing either PMM (10, 20, 40, 80, 120 and 200μM), SIT (10, 15, 20, 30, 50 and 70 μM) or marine extracts at different concentrations. The promastigote assays were performed and ED_{50} & ED_{90} values were calculated as described in chapter 3.

Drug sensitivity of *L. donovani* parasites as intracellular amastigotes was assessed towards PMM (1, 3, 10, 30 and 50 μM) and SIT (1, 3, 10 and 20 μM) as described in chapter 3. In a similar manner, 5 most potent marine extracts (determined from promastigote assay) were further investigated at amastigote stage for their antileishmanial potential. The drugs/extracts used in the study did not pose any cytotoxicity towards J774A.1 macrophage at the concentrations used in assay.

Measurement of nitric oxide accumulation

J774 A.1 macrophage cells were plated at $2X10^5$cells per well in 24-well plates and infected with LdAG83 (1 macrophage:10 parasites). After 24 h, non-internalized parasites were removed by washing with chilled RPMI medium. Uninfected/infected macrophage were then cultured in medium alone, medium containing lipopolysaccharide (LPS) from *E.coli* at 1 μg/ml or different doses of PMM (1-30μM) or SIT (0.5-10μM) at 37 °C under 5% CO_2 for 48 h (in triplicate) with or without prior treatment with 1 mM N-methyl-L-arginine monoacetate (L-NMMA), a competitive inhibitor of nitric oxide synthase (NOS) that inhibits conversion of L arginine to NO. SAG was used as the reference drug to serve as positive control. After 48 h, cell supernatants were collected and stored at -70 °C until nitrite estimation. NO levels were estimated by reducing the nitrate accumulated over 48 h to nitrite with nitrate reductase and measuring the nitrite concentration colorimetrically by Griess reaction. Briefly, the plates were centrifuged at 1300 rpm for 5 min at room temperature and supernatants were collected. 100μl of 40mg/ml of Griess reagent was added to 100μl of cell free supernatant in 96 well plate along with the medium blank and absorbance was measured at 540nm following incubation for 15 min at room temperature. The amount of nitrite accumulated was

calculated from a standard curve constructed with different concentrations of sodium nitrite (in a linear range between 10 and 80 μM).

Statistical Analysis

Statistical analysis was performed as described in chapter 3. Additionally, Student's t test was applied for determining the statistical significance of the mean ED_{50} in Nitric Oxide inhibition assays. Statistical significance was accepted as $p<0.05$.

Results

Susceptibility of *L. donovani* isolates to antileishmanial drugs

The susceptibility to PMM and SIT was determined in 20 VL isolates comprising of 7 from LR and 13 from HR region. The clinical profile of VL patients and *in vitro* susceptibility of the isolates to PMM and SIT in comparison with SAG is summarized in Table 4.1.

Table 4.1: *In vitro* susceptibilities of field isolates of *L.donovani* to paromomycin and sitamaquine

Parasite identifier	Sex/age (yr)[a]	District in Bihar (endemicity zone)[b]	Treatment (response)[c]	SAG Susceptibility[d] ED_{50} (μg/ml), amastigote	Paromomycin susceptibility[d] ED_{50} (μM)		Sitamaquine susceptibility[d] ED_{50} (μM)	
					Amastigote	Promastigote	Amastigote	Promastigote
MHOM/IN/83/AG83		—[e]	—[e]	02.1 ± 0.2	2.2 ± 0.1	29.9 ± 1.8	1.7 ± 0.1	21.8 ± 1.7
MHOM/IN/1999/K59	F/21	Vaishali/HR	SAG (NR)	14.7 ± 3.3	4.5 ± 0.2	50.7 ± 0.6	3.7 ± 0.8	18.9 ± 1.6
MHOM/IN/2000/K131	M/22	Saharsha/HR	SAG (NR)	19.4 ± 1.7	5.1 ± 0.1	20.9 ± 1.3	0.9 ± 0.1	12.3 ± 0.8
MHOM/IN/2001/K149	M/20	Saran/HR	AmB (R)	15.7 ± 4.0	2.6 ± 0.2	27.8 ± 2.5	1.7 ± 0.1	16.49 ± 0.1
MHOM/IN/2002/K192	M/24	Saran/HR	AmB (R)	20.3 ± 0.8	4.3 ± 0.4	21.6 ± 1.2	1.4 ± 0.2	16.7 ± 1.2
MHOM/IN/2003/K251	M/11	Saran/HR	ND	11.8 ± 1.3	5.2 ± 0.1	50.4 ± 3.4	3.5 ± 0.1	20.1 ± 2.3
MHOM/IN/2006/K417	F/8	Muzaffarpur/HR	AmB (R)	14.7 ± 0.7	5.5 ± 0.2	23.4 ± 0.6	3.2 ± 0.4	16.4 ± 0.1
MHOM/IN/2006/K429	M/26	Saharsha/HR	AmB (R)	13.8 ± 0.8	4.9 ± 0.6	19.6 ± 0.9	2.2 ± 0.5	18.7 ± 0.6
MHOM/IN/2006/K439	M/16	Muzaffarpur/HR	AmB (R)	12.9 ± 0.1	1.2 ± 0.01	23.7 ± 1.9	2.8 ± 0.2	13.6 ± 1.4
MHOM/IN/2007/K498	F/55	Madhubani/HR	AmB (R)	15.8 ± 0.2	4.1 ± 0.2	17.1 ± 1.2	2.6 ± 0.5	22.6 ± 1.2
MHOM/IN/2007/K508	M/50	Muzaffarpur/HR	AmB (R)	15.3 ± 0.3	4.7 ± 0.2	35.8 ± 0.9	1.7 ± 0.4	20.3 ± 0.8
MHOM/IN/2007/K509	F/4	Madhubani/HR	AmB (R)	16.8 ± 0.3	4.9 ± 0.3	28.4 ± 1.1	2.7 ± 0.4	32.9 ± 1.6
MHOM/IN/2007/K516	F/60	Motihari/HR	AmB (R)	16.5 ± 0.6	3.2 ± 0.2	23.9 ± 0.5	2.6 ± 0.2	15.9 ± 0.3
MHOM/IN/2007/K518	M/19	Chhapra/HR	AmB (R)	14.9 ± 0.4	2.3 ± 0.5	19.1 ± 0.8	3.5 ± 0.3	17.2 ± 0.6
MHOM/IN/1999/K80	F/40	Bhagalpur/LR	SAG (NR)	10.4 ± 2.2	3.0 ± 0.31	52.7 ± 3.5	0.9 ± 0.1	16.9 ± 1.2
MHOM/IN/2000/K111	F/36	Siwan/LR	SAG (R)	5.6 ± 0.6	2.2 ± 0.2	34.8 ± 0.3	2.1 ± 0.1	14.2 ± 0.3
MHOM/IN/2000/K132	F/24	Munger/LR	ND	3.9 ± 0.3	3.5 ± 0.7	31.6 ± 0.3	2.1 ± 0.2	16.0 ± 0.2
MHOM/IN/2000/K133	M/20	West Bengal[f]/LR	SAG (R)	3.5 ± 0.3	3.0 ± 0.3	31.5 ± 0.7	2.3 ± 0.1	17.5 ± 0.1
MHOM/IN/2000/K135	F/45	Gopalganj/LR	SAG (R)	4.2 ± 0.4	4.7 ± 0.8	34.8 ± 0.3	0.9 ± 0.1	13.9 ± 0.1
MHOM/IN/2001/K155	M/14	West Bengal[f]/LR	SAG (R)	2.1 ± 0.3	5.9 ± 0.2	31.6 ± 0.3	0.6 ± 0.1	20.7 ± 1.3
MHOM/IN/2006/K435	M/17	Uttar Pradesh[f]/LR	AmB (R)	11.8 ± 1.4	2.8 ± 0.1	15.7 ± 2.2	1.7 ± 0.3	12.7 ± 2.9

[a] M, male; F, female.

[b] LR and HR: represents, respectively, zones of low resistance and high resistance to antimony

[c] Responses were noted 30 days after treatment with SAG infusions (20 mg/kg of body weight) or with AmB infusions (1 mg/kg of body weight) on alternate days for 1 month. Patients with an absence of fever and with a reduction in spleen size were designated responders (R); others were considered nonresponders (NR), ND –Not determined.

[d] SAG: sodium antimony gluconate; Mean ED_{50} ± standard error from three separate assays. The ED_{50} values for SAG at amastigote stage given here are as reported earlier (Kumar et al., 2009b).

[e]MHOM/IN/83/ AG83 is reference strain *L. donovani* from India, [f] Neighbouring states of Bihar

Susceptibility to Paromomycin

The field isolates exhibited a wide range of susceptibility to PMM at both promastigote and amastigote stages. The ED_{50} (mean±SEM) at promastigote stage ranged from 15.7±2.2 to 52.7±3.5µM while ED_{90} (mean±SEM) ranged from 25.0±0.8 to 153.9±3.1 µM. The mean and median ED_{50} for PMM at promastigote stage were 29.8±2.5 µM and 28.3 µM while mean and median ED_{90} were 71.6 ±7.7 and 69.8 µM respectively. The amastigotes showed higher susceptibility with ED_{50} ranging from 1.2 ±0.01 to 5.9 ±0.3µM, mean = 4.1 ±0.3 µM, median = 4.2 µM (Table 1). Mean ED_{50} of LR region parasites (3.8±0.5 µM) and HR region parasites (4.2 ±0.3 µM) were comparable (p= 0.47) (Fig. 4.2).

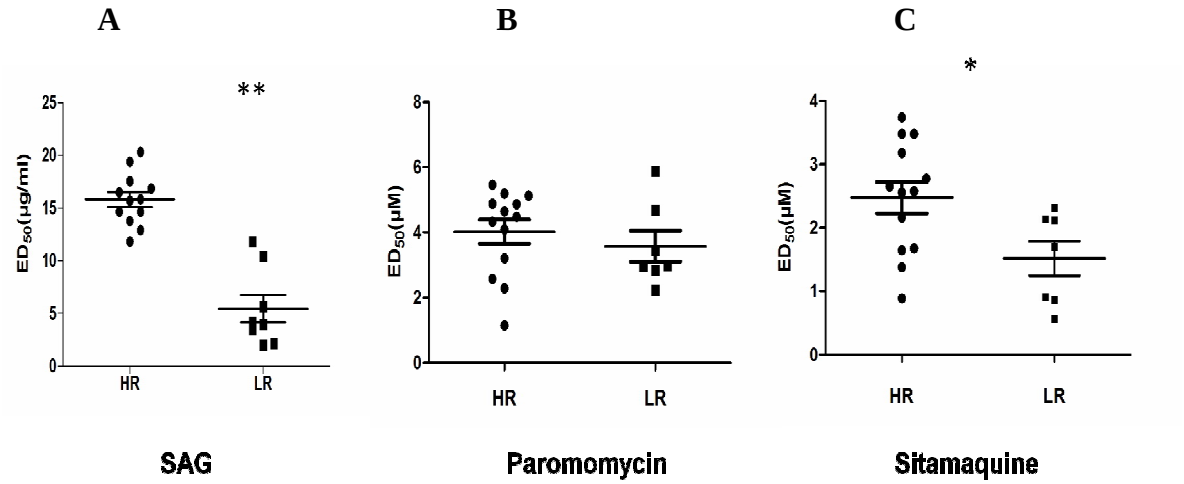
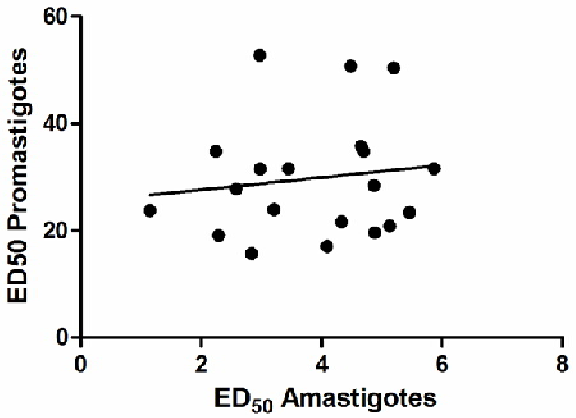


Figure 4.2: Representative plots of susceptibility of parasite isolates from high and low resistance zones towards (A) SAG (B) paromomycin and (C) sitamaquine. ED_{50} of *L.donovani* isolates at intracellular amastigote stage were determined by infection in murine macrophage cell line J774A.1 as described in methods. ED_{50} of VL isolates from high resistance zone (HR) and low resistance zone (LR) towards these drugs are represented by closed circles (●) and closed squares (■) respectively. Horizontal bar represents mean of HR/LR and vertical bars represent the standard error. Each individual

value represents Mean ED_{50} of the results from three separate assays.* ($p<0.05$); ** ($p<0.001$) as determined by Mann Whitney U test.

Antileishmanial activity of PMM did not correlate ($r=0.13$, $p>0.05$) at amastigote and promastigote stages, indicating that intracellular amastigote is the ideal stage for determining the sensitivity towards PMM (Fig. 4.3).



Number of XY Pairs	20
Pearson r	0.1340
95% confidence interval	-0.3281 to 0.5443
P value (two-tailed)	0.5734
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R squared	0.01795

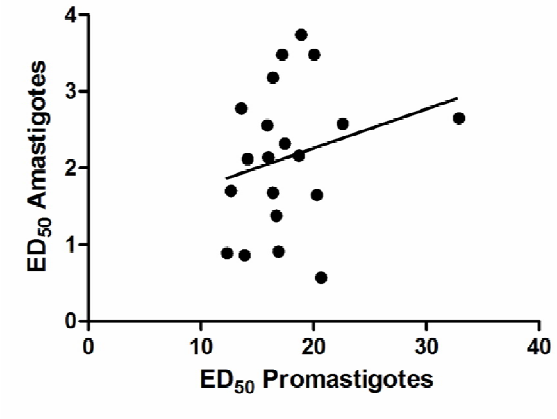
Figure. 4.3: Comparison of paromomycin susceptibility at promastigote and intracellular amastigote stage. The *in vitro* susceptibilities of promastigote and amastigote parasites stages towards PMM did not correlate (r=0.13).

<u>Susceptibility to sitamaquine</u>

VL field isolates displayed a variable susceptibility towards SIT at promastigote stage, with ED_{50} (mean± SEM) ranging from 12.3 ±0.8 to 32.9 ±1.6 µM and ED_{90} (± SEM) ranging from 27.9±1.0 to 62.5±0.7 µM. The mean ED_{50} for SIT was 17.7 ±1.0 µM

and mean ED_{90} was 39.9±2.6 µM (Table 4.1). The median ED_{50} was determined as 16.9 µM and median ED_{90} as 35.8 µM.

Susceptibility at amastigote stage ranged from 0.6 ±0.1 to 3.7±0.8 µM with the mean ED_{50} as 2.1 ±0.2µM and the median ED_{50} as 2.2 µM. Again, the amastigote stage of the parasite was more susceptible in comparison to the promastigote stage, with promastigotes displaying 5 to >35 fold higher ED_{50} (Table 4.1). The isolates from HR region displayed significantly high mean ED50 (p=0.0195) for SIT (2.5±0.2 µM) than those from LR region (1.5 ±0.3 µM) (Fig. 4.2). There was no significant correlation between the susceptibility at the two stages (r=0.26, p>0.05; Fig. 4.4). *In vitro* studies on antiparasitic effect taking total SIT are assumed to be representative of its metabolites which are largely unknown.



Number of XY Pairs	20
Spearman r	0.2634
95% confidence interval	-0.2164 to 0.6406
P value (two-tailed)	0.2619
P value summary	ns
Exact or approximate P value?	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	No

Figure 4.4: Comparison of sitamaquine susceptibility at promastigote and intracellular amastigote stage. The *in vitro* susceptibilities of promastigote and amastigote parasites stages towards SIT did not correlate (r=0.26).

Effect of paromomycin and sitamaquine on production of nitric oxide (NO) by infected macrophages

An increase in NO levels upon PMM or SIT treatment was observed in both uninfected and infected macrophages, the increase being higher in infected cells compared to uninfected cells (Fig. 4.5A). Sodium nitrite dilutions (0-80µM) were prepared using RPMI medium with and without phenol red. 100 µl of the standard dilutions were mixed with equal volumes of Griess reagent followed by 15 min incubation at room temperature and absorbance at 540nm was recorded. Similar experiments were performed for nitrite estimation in uninfected J774 cells exposed to PMM/SIT. The presence of phenol red in medium did not interfere significantly with the nitrite determination by Griess method (not shown).

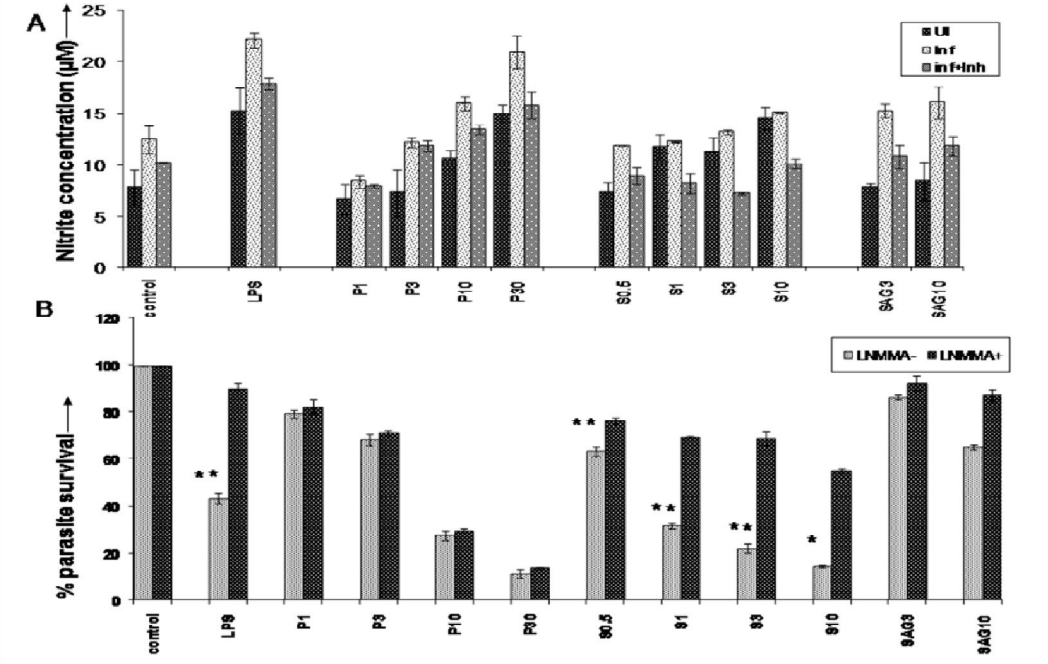


Figure 4.5: Effect of paromomycin and sitamaquine on production of nitric oxide by macrophages and parasite survival. (A) Production of nitric oxide in parasite infected macrophages upon treatment with paromomycin or sitamaquine. J774.A.1 Murine

macrophages uninfected (UI), infected (Inf) with *L.donovani* LdAG83 (1:10 infection ratio) or infected macrophages treated with L-NMMA 1 hr prior to drug exposure (inf+L-NMMA) were incubated with medium alone (control) or medium containing increasing concentrations of paromomycin (P1, P3, P10, P30 denoting 1, 3, 10 and 30 μM respectively) or sitamaquine (S0.5, S1, S3, S10 denoting 0.5, 1, 3 and 10 μM respectively) for 48 h at 37 °C. Lipopolysaccharide (LPS, 1μg/ml) from *E.coli* was used as positive control while SAG was taken as the reference drug, SAG 3 and SAG 10 denoting 3 and 10 μg/ml respectively. Nitrite concentration was measured in supernatants by Griess method. The graphs represent the mean of triplicates from two independent experiments. * ($p<0.05$); ** ($p<0.001$) as determined by t- test. (B) Effect of nitric oxide inhibitor on parasite survival in *L.donovani* infected macrophages treated with paromomycin/sitamaquine. Percent parasite survival in infected macrophages upon treatment with different drugs in presence (L-NMM A+) or absence of inhibitor (L-NMMA^{-}) is shown. Other details same as in panel (A).

Effect of paromomycin on Nitric Oxide release

PMM activated macrophages produced NO in a dose dependent manner and percentage parasite killing correlated significantly with the NO release ($r=0.70$, $p<0.05$) (Fig. 4.5A). Further, upon addition of N-LMMA (NOS inhibitor), a decrease in NO production comparable to the control infected cells was observed, while percent parasite killing did not alter significantly ($p= 0.186$) suggesting that the activity of PMM against *L. donovani* is not dependent on NO (Fig. 4.5B).

Effect of sitamaquine on NO release

SIT treatment induced NO production in a dose dependent manner in infected macrophages. Percentage parasite killing correlated positively with the NO release ($r=0.58$, $p<0.05$). Addition of N-LMMA, resulted in a decrease in NO production comparable to the control infected cells in SIT treated macrophages with significant

decrease in the parasite killing (p= 0.019) suggesting that the cytotoxic activity of SIT against *L. donovani* involves NO pathway (Fig. 4.5B).

Evaluation of Potential of natural products from marine organisms as antileishmanial drug candidates.

25 methanolic extracts were screened for their antileishmanial properties at promastigote stage. Out of these, 12 extracts showed potent antileishmanial activity with ED_{50} < 1mg/ml (ED_{50} ranging from 2.2±0.50 µg/ml to 829±13.28 µg/ml) (Table 4.2).

Table 4.2: Sensitivity of *L.donovani* promastigotes towards marine extracts

S.No:	Organism	ID	Genus	ED_{50} ± SEM (µg/ml)
1	Sponge	**M21**	*Haliclona sp(1)*	**002.20±0.50**
2	Sponge	**M24**	*Oceanapia sp*	**024.68±1.89**
3	Sponge	**M20**	*Axinella sp*	**031.41±6.10**
4	Mollusk	**M23**	*Pleurobranchus sp*	**061.20±4.42**
5	Sponge	**M02**	*Spirastrella sp*	**238.12±15.8**
6	Sponge	**M08**	*Haliclona sp (2)*	**289.44±14.2**
7	Sponge	**M01**	*Sigmadocia sp*	**415.21±08.1**
8	Ascidian	**M15**	*Herdmania sp*	**537.90±13.7**
9	Sponge	**M16**	*Echinodictyum sp*	**584.17±8.42**
10	Sponge	**M10**	*Ircinia sp*	**646.24±13.1**
11	Sponge	**M03**	*Callyspongia sp*	**778.88±12.9**
12	Sponge	**M14**	Unidentified	**829.10±13.28**

- LdAG83, standard *L.donovani* strain used for the study
- Marine extracts for which LdAG83 exhibited ED50<1mg/ml are reported here.
- ED_{50} of 5 most potent extracts showing antileishmanial activity are highlighted
- Spp. not revealed due to IPR

After the initial screening, the 5 most effective extracts with ED_{50} less than 250 µg/ml were selected further for determination of their effectiveness at intracellular amastigote

stage after determining their cytotoxicity towards J774A.1 mouse macrophage cell line. The ED_{50} of these 5 extracts ranged from 2.2±0.50 µg/ml to 238±15.8 µg/ml (Table 4.3).

Table 4.3: Sensitivity of *L.donovani* towards 5 most potent marine extracts

S.No:	**Marine organism ID**	**Organism**	**Promastigote** ED_{50}± SEM (µg/ml)	**Amastigote** ED_{50}± SEM (µg/ml)
1.	**M21**	Sponge	2.20±0.50	1.91±0.80
2.	**M20**	Sponge	24.68±1.89	2.49±0.61
3.	**M24**	Sponge	31.41±6.1	3.59±0.82
4.	**M23**	Mollusk	61.20±4.42	17.71±3.65
5.	**M2**	Sponge	238.12±15.8	43.95±8.27

- § LdAG83, standard *L.donovani* strain used for the study
- § Marine extracts for which LdAG83 exhibited ED_{50} <250 µg/ml are reported here.

Discussion

The present investigation reports for the first time the natural susceptibility of *L. donovani* from Indian VL cases towards upcoming antileishmanial drugs, PMM and SIT. The study reports the scenario of drug susceptibility in the parasite population currently prevailing in the field and as yet unexposed to these drugs. In the present study, more than five fold variations were observed in the *in vitro* susceptibility within field isolates towards these two drugs. The variability in drug susceptibility observed among field

isolates reflects differences in intrinsic susceptibility that could have an important impact on clinical outcome in future.

The sensitivity of PMM and SIT has been previously reported only in reference strains of *Leishmania* (Seifert & Croft, 2006; Bories et al., 2008; Lopez-Martin et al., 2008; Jhingran et al., 2009). The ED_{50} of *L. donovani* for SIT has been reported to be ranging from 19.8-29.2 µM in the promastigote form (Duenas-Romero et al., 2007; Lopez-Martin et al., 2008) and 4.78–5.41 µM in intracellular amastigotes (Seifert & Croft, 2006). Similarly, susceptibility to PMM in *L donovani* ranged from 6- 50 µM at promastigote stage and from 8–48.81 µM at intracellular stage (Jhingran et al., 2009; Seifert et al., 2010).

All VL isolates examined in the study, whether from HR or LR region, were found susceptible to both PMM and SIT. In case of SIT, HR region isolates showed significantly lower susceptibility compared to LR isolates, similar to the previous observation with miltefosine and amphotericin B (Kumar et al., 2009b). On the contrary, PMM exhibited similar sensitivity towards parasite isolates from HR as well as LR zones indicating its potential efficacy in VL endemic area. PMM is a polycationic hydrophilic sugar that specifically possesses high affinities for certain portions of RNAs, and interferes with protein synthesis (Kotra et al., 2000). Its antileishmanial action involves change in mitochondrial membrane potential, ribosomal and respiratory dysfunction, decreased membrane fluidity, leading to altered drug uptake (Jhingran et al., 2009; Maarouf et al., 1997). Comparable PMM sensitivity in parasite population from HR and LR region further points, that it has a distinct mechanism of action from other antileishmanial drugs currently being used.

Leishmanicidal action of antimonials involves generation of reactive oxygen and NO species and affects the MAP kinase pathway (Mookerjee et al., 2006). Insight into mechanism of action of PMM highlights the role of mitochondrial membrane potential, ribosomes and respiratory dysfunction. However, much less is known in regard of mechanism of antileishmanial action of SIT. The present study revealed an increase in NO levels in *L. donovani* infected macrophages upon SIT and PMM treatment, coupled with a marked decrease in the number of amastigotes. Presence of NO inhibitor compromised the amastigote killing by SIT suggesting that its cytotoxic activity against *L. donovani* amastigotes involves NO pathway. Another well established aminoquinoline, chloroquine, was shown to induce the expression of inducible nitric oxide synthase and NO production in C6 glioma cells (Chen et al., 2005).

We observed that the amastigotes were invariably more sensitive to PMM and SIT, up to 4-40 fold and 5-35 fold, respectively. Higher PMM and SIT susceptibility of intracellular amastigotes than promastigotes, like in case of SAG, is suggestive of stage specific antileishmanial action of these drugs. Although previous studies have mainly used *Leishmania* promastigote model for determining the drug sensitivity *in vitro*, lack of correlation between the promastigotes and amastigote susceptibility for both PMM and SIT observed in the present study established the intracellular amastigote model as more appropriate for susceptibility studies as it has been reiterated for other antileishmanial reference drugs (Vermeersch et al., 2009).

PMM appears promising since (i) SAG resistant or sensitive field isolates were equally susceptible and (ii) it has a distinct mechanism of action compared to other currently available antileishmanials. In this study, we have established that isolates from

SAG responsive and unresponsive patients are susceptible to PMM and SIT. Additionally, PMM having a different mechanism of action to existing antileishmanials would be a useful candidate in combination therapy.

Marine biota can be an ideal resource for exploring potent chemical entities to be used in drug discovery as the marine organisms produce molecules with unique structural features to survive in the adverse conditions of the marine environment (Jain et al., 2008). In recent years, marine natural product research has yielded a considerable number of drug candidates (Fournet & Munoz, 2002; Kayser et al., 2003). Several agents effective against chronic pain, Alzheimer's disease, cancer asthma, etc. have been developed and are currently under preclinical or early clinical development (Haefner, 2003). In the present study, crude methanolic extract from marine sponges, ascidians and mollusks showed considerably good inhibitory activity against both promastigote and intracellular amastigote forms of *L. donovani*. Four species of marine sponge and a mollusc showed potent antileishmanial activity, of which the highest efficacy was exhibited by sponge *Haliclona.* The *in vitro* efficacy of the crude extracts of *Haliclona* was comparable to that of standard antileishmanial drugs. Studies have demonstrated the antileishmanial potential of marine red algae, *Asparagopsis taxiformis* (Genovese et al., 2009). Efficacy of *Haliclona* extract has been reported earlier towards *L. donovani* (Dube et al., 2007) and filaria (Lakshmi et al., 2009). *In vivo* antitumor activity and other pharmacological properties of halitoxin obtained from the sponge *Haliclona viridis* have also been demonstrated (Baslow & Turlapaty, 1969).The amino alcohol fraction from *Haliclona* spp. has been shown to act as an antifungal agent and inhibit the development of ascidian *Herdmania curvata* (Clark et al., 2001). It will be of interest to find active ingredient

from the potent marine extracts identified in the study to provide new lead toward development of an effective antileishmanial agent for exploiting the vast world of marine resources to combat the parasite. The study therefore provides an initial rationale for the evaluation of marine compounds and other similar natural products as alternative or possibly synergistic compounds for current antileishmanial activity.

Chapter 5

Generation and Characterization of experimental Leishmania donovani strain resistant to Miltefosine

Introduction

The ever increasing failure of SAG therapy in kala azar (KA) endemic regions urged the authorities of India, Nepal and Bangladesh to replace the current treatment regime with the oral antileishmanial drug Miltefosine (MIL), which constitutes one of the major pillars of the kala-azar elimination programme in the Indian subcontinent (Chappuis et al., 2007; Joshi et al., 2008). MIL has also been reported to be highly effective for PKDL therapy in India (Sundar et al., 2006; Ramesh et al., 2011) where due to prolonged treatment course (almost 3-4 times as that in VL), other drugs depict limitations of toxicity and noncompliance. However, long treatment course and long half-life indicate that MIL resistance could develop quickly. Since directly observed therapy (DOT) has still not been implemented for MIL treatment in India, widespread misuse of this self administered drug could contribute to rapid emergence of MIL resistance in field. Moreover, phase IV trial of MIL in India has suggested doubling of the relapse and failure rate compared to phase III trials (Sundar & Murray., 2005; Sundar & Rai, 2005). Reports on MIL treatment failures (almost all relapses) were also observed in Nepal (Pandey et al., 2009). In this situation, it becomes very crucial to understand the mechanism of MIL resistance, which is poorly understood till date. In experimental models, the anti-leishmanial activity of MIL has been demonstrated against all stages of the *L. donovani* (Croft et al., 1987; 1996; Kuhlencord et al., 1992). Various mechanisms of action of MIL against tumour cells have been identified, including inhibition of enzymes of cell signaling pathways and induction of apoptosis (Henke et al., 1999). Mechanism(s) of action of MIL against *Leishmania* and the closely related parasite *Trypanosoma cruzi* so far identified include effects on perturbation of

the alkyl-lipid metabolism, phospholipid biosynthesis and membranes (Lira et al., 2001; Lux et al., 2000; Santa-Rita, 2000). In *Leishmania,* a P-glycoprotein-mediated resistance to ether lipids has been described in *L. tropica* (Perez-Victoria et al., 2001).

To investigate further the anti-leishmanial mechanisms of action of MIL, in the present study, *L. donovani* promastigote resistant lines were generated in lab. We further evaluated the phenotypic and genotypic characteristics of experimental MIL resistant *L.donovani* based on its growth profile, altered susceptibility to MIL, susceptibility to other antileishmanial drugs, infectivity or virulence compared to wild type parasite. Earlier reports on experimental MIL resistance indicated impairment in activity of P-Type ATPase transporters, LdMT–LdRos3 dependent flippase machinery at the plasma membrane leading to decreased drug uptake and therefore, a reduced susceptibility to MIL (Perez-Victoria et al., 2003; 2006a). We therefore evaluated the mRNA expression of LdMT and LdRos3 in the MIL resistant parasite. The study sets the ground for the differential gene expression profiling using a well characterized MIL resistant parasite.

Materials and methods

Parasites

Based upon the MIL sensitivity profile of *L. donovani* parasites described earlier, one isolate (K417), depicting intrinsic high tolerance to MIL *in vitro* was selected for development of MIL resistant mutant. K417 parasite was isolated from KA case hailing from hyper endemic region of Bihar, India, and the parasite was characterized as *L.donovani* by species specific PCR (Salotra et al., 2001). The parasite line, referred to as

L. donovani Wild Type (WT) was maintained in M199 medium supplemented with 40 mM HEPES, containing 10% fetal calf serum (FCS) at 26°C.

Development of Miltefosine resistant lines

Experimental *L. donovani* strain resistant to MIL was obtained by growing promastigotes *in vitro* with step-wise increase in the drug pressure (Seifert et al., 2003). WT parasites (5 X 10^5 cells/ml) were initially exposed to 2.5 µg/ml MIL (M199 + 10 % FCS) and subsequently, in stepwise fashion, to 5, 10, 20 and 30 µg/ml concentration of MIL. At each step, parasites were cultured for at least 5-8 passages to attain steady and optimal cell growth. A stepwise increase in drug concentration was undertaken only when the drug exposed cultures showed a growth rate equivalent to that of the wild-type clone cultures. Resistant clones M2.5, M5, M10, M20 and M30 were also stored in liquid N2 at various stages during their establishment. The growth profile of the MIL resistant parasite (referred as LdMIL-R) was compared with its corresponding wild type parasite.

***In vitro* infectivity**

The infectivity of MIL-resistant promastigotes was assessed using a murine macrophage J774A.1 cell line. The promastigote forms of WT and MIL-R parasites were used for macrophage infection from 6 day old cultures. The macrophages were infected with WT and MIL-R promastigotes as described in chapters 3 and 4. Infected macrophages were incubated for 24, 48 or 72 hrs at 37°C at 5% CO_2 and slides were stained with Diff-Quik solution. The number of amastigotes in 100 macrophages/ well and the mean number of amastigotes/macrophage were determined microscopically as described earlier.

Susceptibility of MIL-R parasite to antileishmanial drugs

Promastigotes assays were performed in WT/MIL-R parasites as described in chapters 3 and 4. Similarly, amastigote assays towards MIL (5-30µg/ml) were performed in J774A.1 cell lines with WT/MIL-R parasites as described previously. The fold decrease in MIL susceptibility of the MIL resistant parasite was taken as the ratio of ED_{50} of MIL-R to the ED_{50} of corresponding wild type parasite (Fig 5.1).

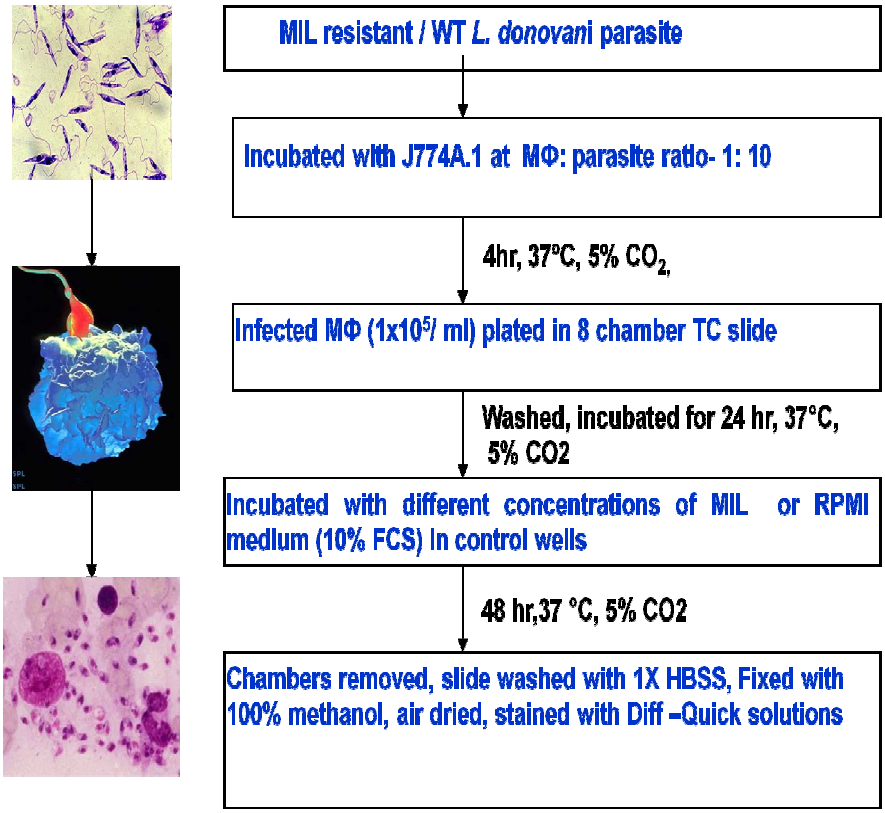


Figure 5.1: Methodology of intracellular amastigote assay. The MIL resistant parasites were evaluated for any alteration in infectivity or virulence compared to its corresponding wild type parasite.

Stability of resistance

Following the establishment of the above resistant lines, cultures of MIL-R *L. donovani* were removed from drug pressure. At 2 weeks, 4 weeks and 12 weeks after removal from drug pressure, the ED_{50} values of cultures were re-determined as described in chapter 3.

Cross-resistance

The determination of cross-resistance to other antileishmanial drugs (SAG, AmB, PMM, and SIT) was carried out using *L. donovani* MIL-R parasites at both promastigote and intracellular amastigote stage and ED_{50} and ED_{90} values were determined as described in chapters 3 and 4.

mRNA expression analysis of LdMT and LdRos3 genes

The gene expression levels of LdMT and LdRos3, reported markers of MIL resistance, were determined for MIL-R *L. donovani* parasite in comparison with the expression levels seen in the corresponding wild type (K417) parasites. All Real time PCR reactions were performed as described in chapter 3. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from real time quantitative PCR experiments.

Results

Development of MIL resistant Parasite

K417 *L. donovani* parasite (LdWT) was exposed stepwise to increasing MIL pressure up to 30µg/ml and stable MIL resistance could be achieved in 32 weeks. There was no significant difference in the morphology of MIL-R parasites in comparison with WT.

Growth pattern of LdMIL-R parasite

The resistant parasites exhibited growth kinetics similar to the wild type parasite The MIL-R parasite exhibit similar growth profile in the presence or absence of 30 µg/ml MIL pressure (Fig 5.2).

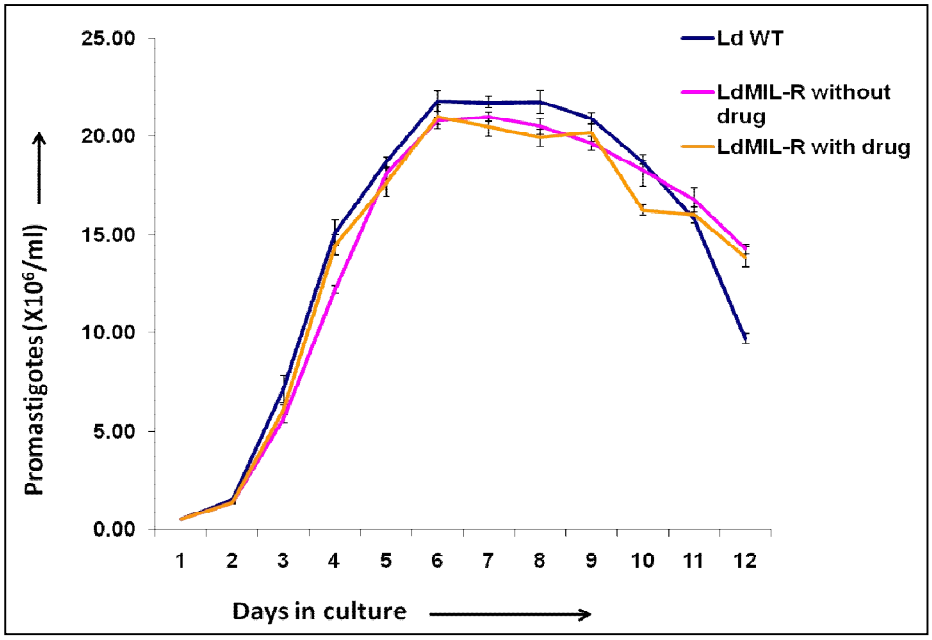


Figure 5.2: Growth profile of cultured MIL resistant and wild type promastigotes in routine conditions for 12 days. LdWT- *L.donovani* wild type parasite, LdMIL-R- *L.donovani* parasite resistant to 30 µg/ml MIL. Error bars indicate standard error of the mean (SEM) based on 3

repeated experiments. The MIL-R parasite exhibited a similar growth profile to WT in the presence or absence of 30 µg/ml MIL pressure.

In vitro infectivity studies of MIL-R parasites

The ability of the MIL-R line to infect J774A.1 macrophages was assessed in comparison to those of the WT clone. This experiment mimics the initial stages of infection of the macrophage after transmission of *Leishmania* from the sandfly to the host. At the early stage of infection (24 hours post infection), MIL-R parasites showed a comparable infection index compared to wild type parasite (p=0.34).The infection index of both the parasites was similar at 48 and 72 hrs post infection (Fig 5.3A) and the percentage of infected macrophages at 72 hrs was comparable with 83.2 ± 4.4% for WT and 78.3± 5.2% for MIL-R parasites, and the mean number of amastigotes/macrophage was 4.5± 1.1 and 4.7± 0.4, in WT and MIL-R, respectively. The Diff-Quik stained MIL-R /WT infected macrophages as observed at 100X magnification under oil immersion are depicted in Fig. 5.3 B and C.

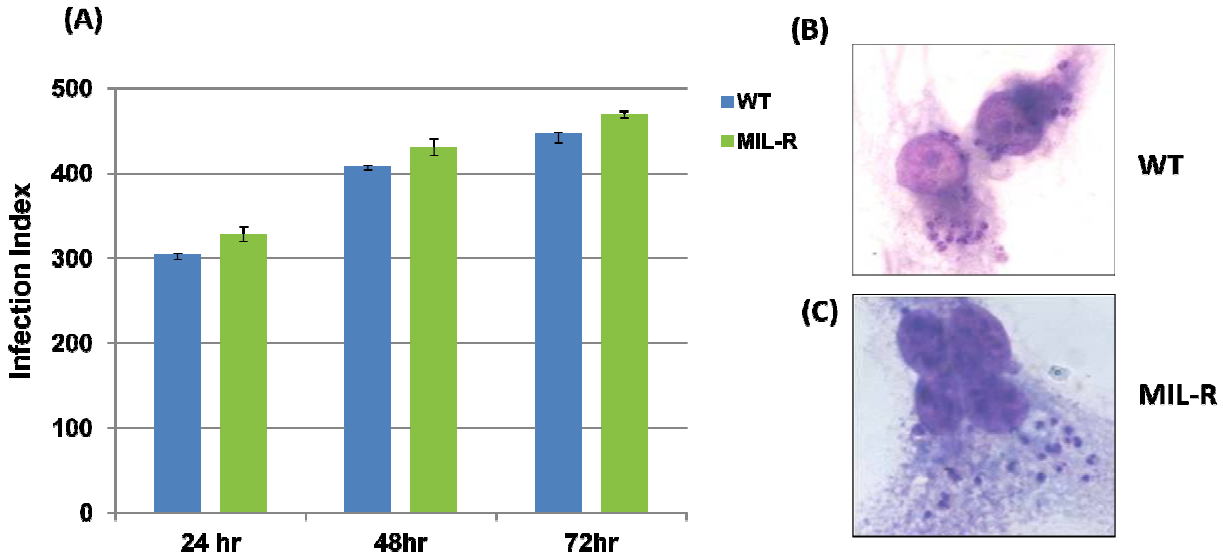


Figure 5.3: **Infection index of MIL-R and WT parasites at 24, 48 and 72 hours post infection.** (A) Infection index is defined as the number of amastigotes in 100 macrophages. Error bars indicate the standard error of the mean based on 3 repeated experiments. Comparable infectivity in (B) Diff-Quik stained wild type infected J774A.1 macrophages and (C) MIL-R infected J774A.1 macrophages as determined at 72hrs.

MIL susceptibility of MIL resistant parasite at promastigote stage

Following establishment of resistance, sensitivity of MIL-R parasite to MIL was determined. The resistant parasite displayed 18.5 fold higher resistance to MIL (mean ED_{50}± SEM= 64.38 ± 2.17mg/ml) compared to its corresponding wild type parasite (Mean ED_{50} 3.48 ± 0.18 mg/ml) (Fig 5.4, Table 5.1).

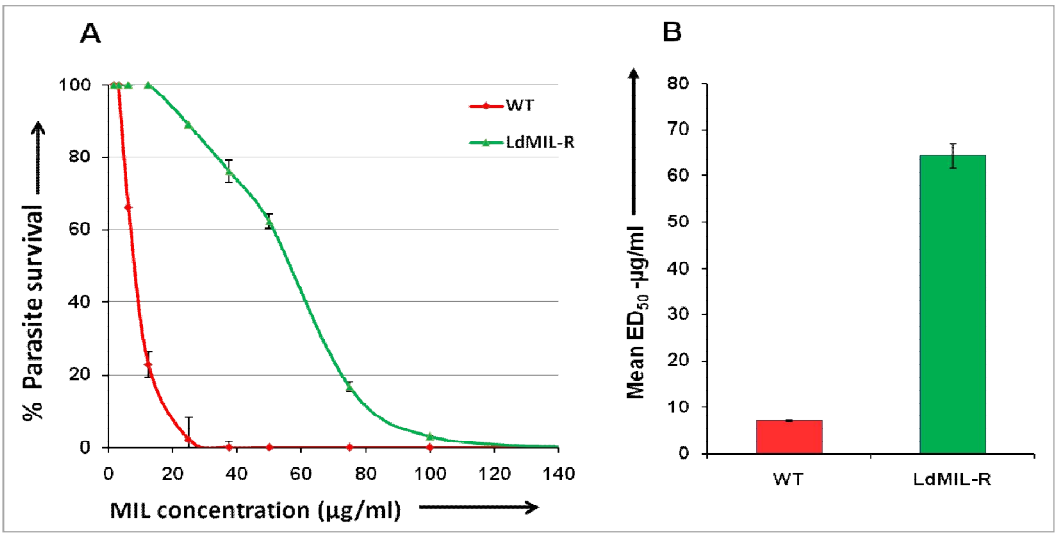


Figure 5.4: **Susceptibility of Miltefosine adapted parasite at promastigote stage.** (A) Dose response curve of the MIL-R parasite (LdMIL-R) compared to sensitive parasite (WT). (B) The mean ED_{50} ±SEM (µg/ml) of the MIL-R parasite compared to WT clearly indicates that the MIL adapted parasites showed a high tolerance at promastigote stage.

MIL sensitivity of MIL-R parasite at amastigote stage

The MIL resistant parasite displayed the mean ED_{50} of > 30mg/ml at intracellular amastigote stage. The drug sensitivity could not be determined above this concentration as MIL poses cytotoxic effect on the host macrophages above the concentration of 30mg/ml. The tolerance to MIL was more than 7.86 fold higher in comparison to its Wild type (WT) counterpart (Fig 5.5).The results indicate that the resistance to MIL induced at the promastigote stage was also evident at the amastigote stage of the adapted parasite.

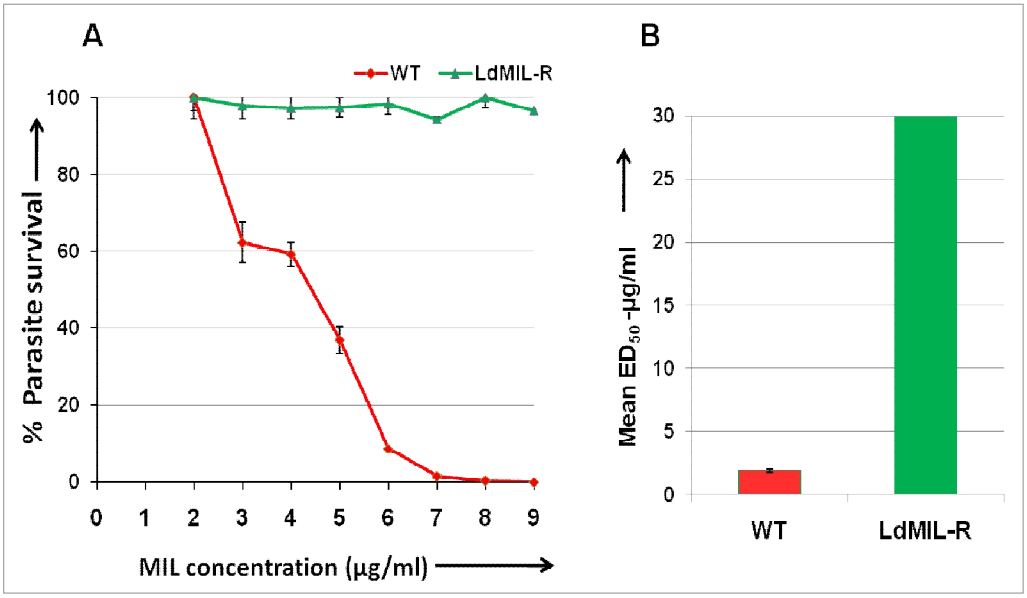


Figure 5.5: **Susceptibility of Miltefosine adapted parasite at amastigote stage.** (A) Dose response curve of the MIL-R parasite (LdMIL-R) compared to sensitive parasite (WT). (B) The mean ED_{50} ±SEM (µg/ml) of the MIL-R parasite compared to WT clearly indicates that the MIL resistance induced at promastigote stage was evident at amastigote stage as well.

Evaluation of Cross-resistance to other antileishmanial drugs for MIL resistant parasite

We evaluated the susceptibility of MIL resistant parasite LdMIL-R towards other antileishmanial drugs (SAG, AmB, PMM and SIT) at intracellular amastigote stage to determine if there is any alteration in the parasite susceptibility to these drugs compared

to wild type parasite. The comparative sensitivity of the WT clone and MIL-R line to antileishmanial agents is reported in Table 5.1. Cross-resistance indices were determined by calculating the ratios of the ED_{50} values of resistant lines to the WT. A drug was only considered to be cross-resistant when an index over 5 was consistently determined; a two/three-fold variation in ED_{50} values between drug assays is common. No cross-resistance was found towards other antileishmanial drugs in this study in the resistant parasite population (Table 5.1).

Table 5.1: *In vitro* susceptibility of MIL adapted parasites towards other antileishmanial drugs

Antileishmanial drug	**ED_{50} ± SEM Wild Type**	**ED_{50} ± SEM MIL resistant**	**Cross resistance Index (CRI)**
SAG (µg/ml)	**14.65 ± 0.67**	**17.76 ± 1.38**	**1.2**
AmB (µg/ml)	**0.44 ± 0.01**	**0.52 ± 0.14**	**1.2**
SIT (µM)	**3.18 ± 0.38**	**2.65 ± 0.45**	**0.8**
PMM (µM)	**5.46 ± 0.20**	**7.64 ± 0.15**	**1.4**

Foot note: ED_{50} values represented are the mean of two independent experiments performed in triplicate. Cross-resistance indices determined as ratios of ED_{50} of resistant lines to the WT.

mRNA expression of LdMT in Miltefosine adapted parasite

The gene expression levels of LdMT as markers of MIL resistance were determined in the MIL resistant parasite. The MIL-R parasite exhibited 7 fold down regulated expression of LdMT compared to Wild Type parasite. With respect to LdAG83, the MIL adapted parasite exhibited 25 fold down regulated expression of LdMT (Fig. 5.6).

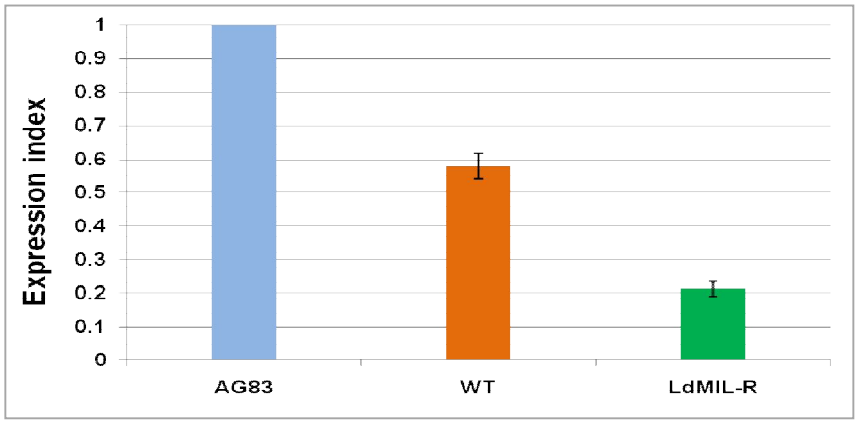


Figure 5.6: **mRNA expression of LdMT in Miltefosine adapted parasite**. The expression pattern of LdMT in MIL adapted parasite. Gene Expression was analyzed using Real Time PCR. Expression index (EI) of gene in various field isolates is indicated with respect to LdAG83 (EI taken as 1).Values given are mean ± SD of three different experiments performed in duplicate.

mRNA expression of LdRos3 in Miltefosine adapted parasite

The MIL-R parasite exhibited 2.51 fold down regulated expression of LdRos3 in MIL resistant parasite compared to WT. With respect to LdAG83, the MIL adapted parasite exhibited 6 fold down regulated expression of LdRos3 (Fig. 5.7).

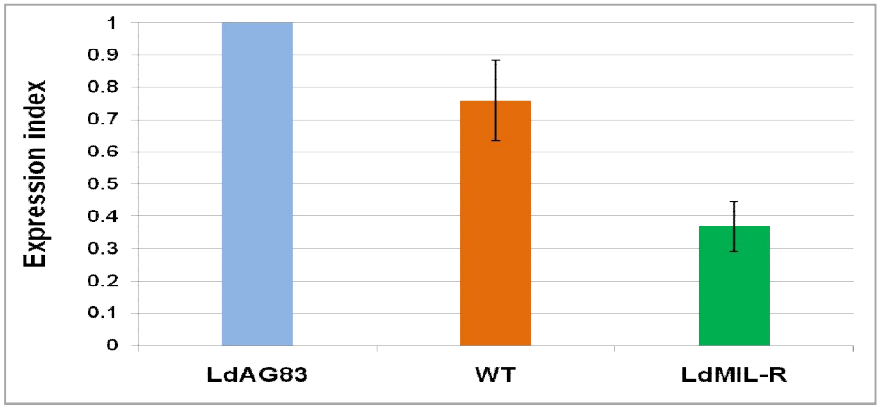


Figure 5.7: **mRNA expression of LdRos3 in Miltefosine adapted parasite**: The expression pattern of LdRos3 in MIL adapted parasite. Gene Expression was analyzed using Real Time PCR. Expression index (EI) of gene in various field isolates is indicated with respect to LdAG83 (EI taken as 1).Values given are mean ± SD of three different experiments performed in duplicate.

mRNA expression of LdMT in the intermediate stages of MIL adapted parasite

The gene expression analysis of LdMT and LdRos3 was carried out in the intermediate stages of MIL adapted parasite to understand how the expression changes as MIL resistance increases. LdMT exhibits significantly down regulated expression above ED_{50} of ~ 19 µg/ml MIL (Fig 5.8) while LdRos3 exhibits significantly down regulated expression above ED_{50} of ~ 25 µg/ml MIL adaptation in the intermediate stages (Fig. 5.9).

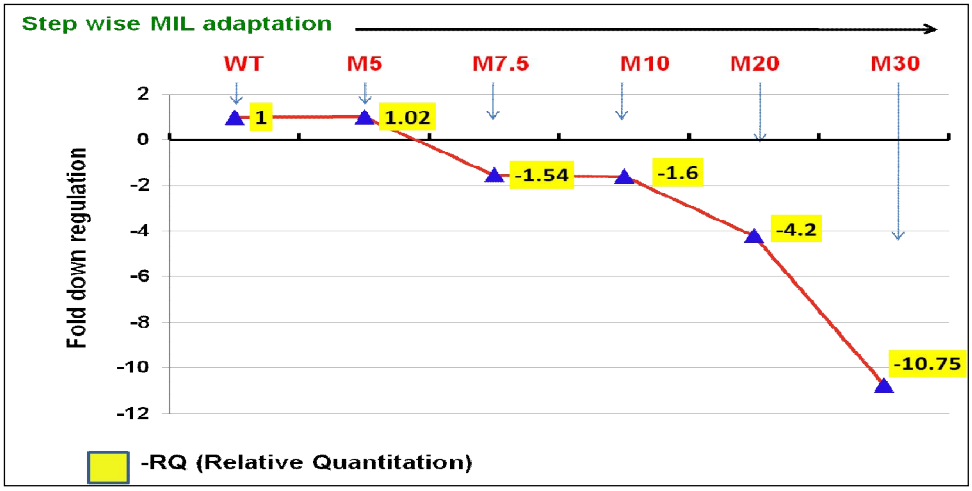


Figure 5.8: mRNA expression of LdMT in the intermediate stages of MIL-R parasite. Relative Quantification (RQ) of gene at different stages of MIL adaptation of MIL-R parasite is indicated in yellow which represents the fold modulation with respect to wild type, WT. Data represents mean ± SD of 2 different experiments performed in triplicate.

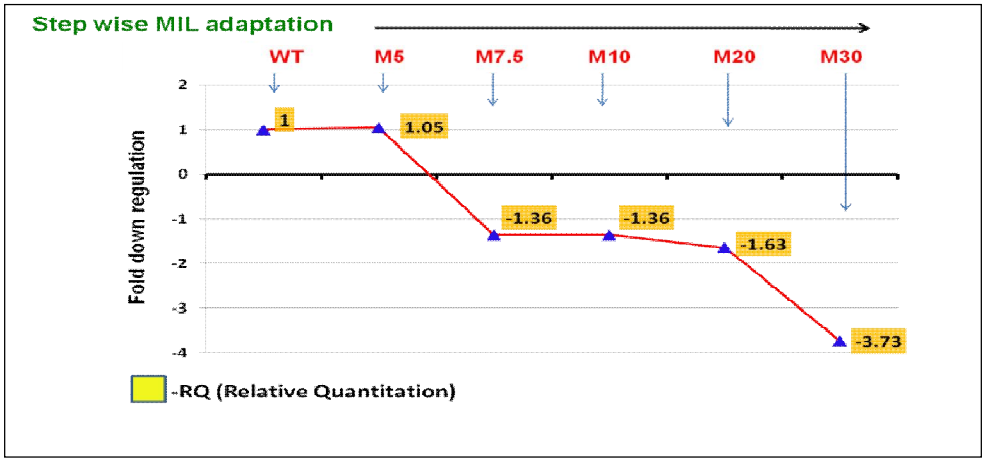


Figure 5.9: mRNA expression of LdRos3 in the intermediate stages of MIL-R parasite. RQ of gene at different stages of MIL adaptation of MIL-R parasite is indicated with respect to wild type, WT. Data represents mean ± SD of 2 different experiments performed in triplicate.

Discussion

Due to the high toxicity and reported failure of antimony treatment, miltefosine is now the official first line treatment against VL in the Indian subcontinent. Factors related to the emergence and spread of drug resistance in VL in India are (i) improper use and mishandling of the drug and (ii) intensive transmission of anthroponotic *L. donovani* (Croft et al., 2006). Certainly, the same factors could set the scene for rapid emergence of MIL resistance in India as the drug is too expensive for impoverished patients to buy a full course treatment (Olliaro et al., 2005; Sundar & Murray, 2005) and additionally, has a long half-life. In an attempt to ensure its longest life as an antileishmanial drug, it is necessary to anticipate the phenomenon leading to a possible failure of its efficacy. Hence, the studies focused on understanding the mechanisms that permit *Leishmania* to resist the effects of MIL are paramount that can then help develop new strategies to overcome the resistance. In the present study, *L. donovani* promastigote lines resistant to MIL were selected by stepwise increase in drug pressure, as shown with other antileishmanial drugs (Al-Mohammed et al., 2005; Bories et al., 2008). Resistant lines were generated up to a concentration of 30µg/ml MIL *in vitro*. The selection of the MIL-R could be achieved in about 32 weeks. It is a stable resistant line since it maintained MIL resistance even after stopping drug pressure for upto 12 weeks. This stability of the MIL resistance is an alarming data that could happen in the field. Similar strategy has been applied in earlier studies to generate experimentally resistant parasites in lab that were successfully employed for studies on understanding the mechanism of resistance in the parasite towards antimony (Singh et al., 2010), MIL (Seifert et al., 2003), AmB (Al-Mohammed et al., 2005) and SIT (Bories et al., 2008) .

Several studies on prokaryotes and eukaryotes have demonstrated that drug resistance generally confers a reduction in fitness expressed as reduced growth, virulence or transmission (Hastings & Donnelly, 2005; Andersson & Hughes, 2010). In some cases, compensatory mutations may occur which restore the fitness of mutants to that of sensitive forms (Walliker et al., 2005). The data from the current study suggests that the growth profile and infectivity of the MIL resistant parasite is similar, suggesting restored virulence in these parasites since parasite load was found comparable in murine macrophages *in vitro*. In the field, resistant parasites fit to establish infections, could lead to rapid transmission of a MIL resistant population and put the lifespan of this important drug at risk. The resistant lines showed up to 18 times higher ED_{50} values for MIL than the WT at promastigotes. The MIL resistance induced at the promastigote parasite stage was evident at the amastigote stage which exhibited about 8 fold higher ED_{50} than WT counterpart.

A number of studies have characterized *in vitro* tumour lines resistant to MIL (Zoeller et al., 1995; Fleer et al., 1996) and shown reduced drug uptake and increased rate of drug metabolism (Fleer et al., 1996), P-glycoprotein over-expression (Rybczynska et al., 2001), elevated levels of bcl-2 (Fu et al., 1999) and increased cholesterol content of plasma membrane (Diomede et al., 1993). The involvement of a P glycoprotein- like transporter in alkyl lyso-phospholipids resistance was previously characterized in a multidrug resistance *L. tropica* line (Perez Victoria et al., 2001). The involvement of LdMT, a P glycoprotein transporter and LdRos3, its beta subunit, in alkyl lysophospholipids resistance was previously characterized in a MIL resistance *L. donovani* line (Perez Victoria et al., 2006a). MIL-R parasite generated in the study

exhibited down regulated expression of these reported MIL-R markers, LdMT and LdRos3. Our studies described in chapter 3 demonstrated that the expression of these MIL-R markers did not correlate with MIL susceptibility in field isolates. The current data with intermediate stages of one MIL-R parasite showed that alteration in expression was evident only in highly resistant parasites (ED_{50} values above 19 μg/ml for LdMT and above 25 μg/ml for LdRos3). The ED_{50} of the parasites in field is much below this concentration and that might explain why presently prevailing field isolates do not show an altered expression of these genes. Further, none of the standard antileishmanial drugs displayed cross-resistance in MIL-R strain. Our results are in line with the previous reports where MIL-R parasite was cross resistant only to edelfosine (ET-18-OCH3, also known as methyl-PAF), an alkyl glycerol-phosphocholine and not to any of the standard antileishmanial drugs (Seifert et al., 2003).

This well characterized MIL-R *L. donovani* parasite line is a stable cellular tool to investigate further mechanisms of MIL action and resistance. As the next step, the MIL targets were identified and characterized (as described in chapter 6) using transcriptomic analysis of this MIL-R parasite employing whole genome *Leishmania* microarray technique with the aim to propose molecular markers of MIL resistance that will help to design strategies to combat this resistance phenomenon.

Transcriptome profiling for identification of Miltefosine resistance associated genes of Leishmania donovani

Introduction

Leishmania donovani is the etiological agent of anthroponotic Visceral Leishmaniasis (VL), a potentially fatal systemic protozoal infection in the Indian subcontinent. The emergence and spread of resistance to therapy for VL is of significance in India where more than 60% patients do not respond to traditional antimonial therapy. This fact has changed the strategies for VL control from clinical and epidemiological point of view. Miltefosine (MIL; hexadecylphosphocholine), is an oral drug initially developed as an anticancer agent for the treatment of cutaneous lymphomas and breast cancer, and showed selective activity against *Leishmania* (Clive et al., 1999; Croft et al., 2006). In India, MIL has recently taken over as the first line therapy for VL even in areas where SAG is effective (WHO, 2004). However, widespread use of MIL monotherapy might lead to the rapid emergence of resistance in India, where VL is anthroponotic (Bryceson, 2001). The long half-life of the drug may potentially increase the risk of development of resistance, especially if it is used in incomplete courses and if the relapse cases are not thoroughly treated. Experimental resistance to this drug was shown to be readily induced *in vitro* (Seifert et al., 2003). Concerns have been raised over rise in MIL treatment failure and relapses (almost double) in Phase IV clinical trial in India. Already, reports of clinical failure and relapse have come up in Nepal (Pandey et al., 2009). In this situation; it becomes very crucial to understand the exact mechanism of action of this drug and the resistance mechanism that might arise in future.

Understanding of mechanism of action of alkylphospholipid (APL) such as MIL in cancer cells suggested that, in contrast to classic chemotherapeutic drugs that target the DNA, APLs act at cell membranes by interfering with the turnover and synthesis of

natural phospholipids and by disrupting membrane-signaling networks at multiple sites, leading to cell death (Boggs et al., 1995). Previous studies showed that the human epidermoid tumour KB cell line made resistant to MIL had a slower uptake and faster MIL metabolism compared to sensitive cells, but resistant cells can incorporate higher amounts of MIL without adverse affects on cell growth and viability (Fleer et al., 1996; Small et al., 1997). Several other mechanisms of resistance are known, including expression of the MDR1 (multidrug resistance) gene or the MRP (multidrug resistance protein) gene, altered glutathione metabolism, expression of bcl-2 protein (Fu et al., 1999; Rybczynska et al., 2001).

The mechanisms and related biological pathways that contribute to MIL resistance in the parasite are relatively poorly understood. Suggested targets of MIL in *Leishmania* include perturbation of ether-lipid metabolism, glycosylphosphatidylinositol anchor biosynthesis, signal transduction as well as inhibition of acyltransferase, an enzyme involved in lipid remodeling (Lux et al., 2000). Current evidence suggests that the drug kills *Leishmania* cells by a process reminiscent of programmed cell death (Verma & Dey, 2004). An impairment in drug uptake machinery involving amino-phospholipid translocase MIL transporter (LdMT) and an accessory protein, LdRos3 (CDC50/Lem3 family) in experimental MIL-resistant *Leishmania* lines was proposed to be the most likely mechanism of resistance (Perez-Victoria et al., 2003; 2006b). Proteomic analysis revealed role of elongation factor eIF4 in MIL resistance in *L.donovani* (Singh et al., 2008). Regardless, a better understanding of the molecular mechanism involved is paramount.

The completion of the genomic sequences of several *Leishmania* species (www.genedb.org) provided the opportunity to study the pattern of whole-genome differential expression during drug resistance. In *Leishmania,* gene organization is polycistronic and mostly the regulation of gene expression occurs at post transcript level by trans-splicing and polyadenylation. Regardless of this fact, transcriptome analysis in these parasites holds importance and provides key information on gene expression modulations. In recent times, gene expression microarray has become well established technology by which the expression of hundreds of genes can be measured simultaneously and provide a global genetic perspective on complex biological processes like drug resistance. Various studies have demonstrated the usefulness of whole-genome DNA microarrays for studying drug resistance in *Leishmania* (Ubeda et al., 2008; Leprohon et al., 2009a; Singh et al., 2010). However, the modulations in *Leishmania* transcriptome during MIL resistance are poorly explored.

The current study utilized whole genome *Leishmania* spp. oligonucleotide array to explore differences in gene expression between MIL resistant and sensitive *L. donovani* parasite. In this study, populations of *L. donovani* resistant to MIL were selected *in vitro* in order to study global gene expression modulation associated with MIL resistance. Individual genes with significant changes in expression in MIL resistant *L.donovani* were validated using qRT-PCR. In depth bioinformatic analysis was performed to identify changes in groups of interacting genes or pathways that may contribute to resistance to MIL. We have found evidence of altered expression of several genes and pathways such as ATP Binding Cassette (ABC) transporters and trypanothione metabolism in MIL resistance in *L.donovani* parasite. The study improves

the understanding of the adaptation of *Leishmania* to high MIL pressure leading to resistance. Such knowledge could be helpful in monitoring resistance in clinical isolates, to subsequently target major molecular interactions associated with such resistance, to find interacting drugs able to overcome the MIL resistance phenotype and identify potential biomarkers of MIL resistance.

The development of drug resistance is of tremendous significance to patients, researchers, and health care providers relying on conventional antileishmanial agents for the treatment of VL. Within this context, an insight into the mechanism of resistance towards MIL, the sole available oral antileishmanial agent, through mRNA expression profiling could contribute to the development of efficient strategies for monitoring MIL resistance at sites where VL is endemic and thereby help to improve the therapeutic index of this valuable class of drug.

Materials and methods

Materials

RNA clean up kit (RNeasy) was obtained from Qiagen. RNA marker, DNAse treatment kit and restriction enzymes were from MBI Fermentas & New England Biolabs, USA. Gene specific oligonucleotides were synthesized from Sigma, USA. The custom *Leishmania* oligonucleotide array and Quick Amp Labeling and Hybridization kit was obtained from Agilent Technologies, USA. Source of rest of the reagents is as described in chapter 3.

Parasite culture

The *L. donovani* parasites (MIL-R/WT) were cultured in Medium 199 supplemented with 10% FCS at 260C and 7.4 pH. Promastigote forms of these parasites were maintained in 15 ml sterile conical centrifuge tubes and transferred into fresh medium every 4-5 days, as necessary.

Isolation of RNA

Total RNA was isolated from MIL sensitive and resistant parasites in late log phase (day 6) according to the manufacturer`s instructions as described in Chapter 3. The purified RNA was quantified Nanodrop by checking the absorbance at 260nm and 280nm. The quality and integrity of RNA was checked on RNA 6000 Nano Assay Chips (Bioanalyzer).

Experimental approach

To investigate the global mRNA expression profiles of MIL resistant and MIL sensitive *L. donovani*, gene expression studies were performed between lab generated MIL resistant *L. donovani* (LdMIL-R, adapted to 30ug/ml of MIL, test sample, designated as T1) and its corresponding wild type parasite (LdWTK417, control sample). The experiment was performed using three biological replicates of each. The sample IDs of each of the biological replicates are mentioned below.

SAMPLE ID	CONDITIONS
US91303662_252751110001_S01_GE1_105_Dec08_1_3.txt	T1
US91303662_252751110001_S01_GE1_105_Dec08_1_4.txt	T1
US91303662_252751110001_S01_GE1_105_Dec08_2_1.txt	T1
US91303662_252751110001_S01_GE1_105_Dec08_2_2.txt	Control
US91303662_252751110001_S01_GE1_105_Dec08_2_3.txt	Control
US91303662_252751110001_S01_GE1_105_Dec08_2_4.txt	Control

The workflow for sample preparation and array processing is explained in fig 6.1.

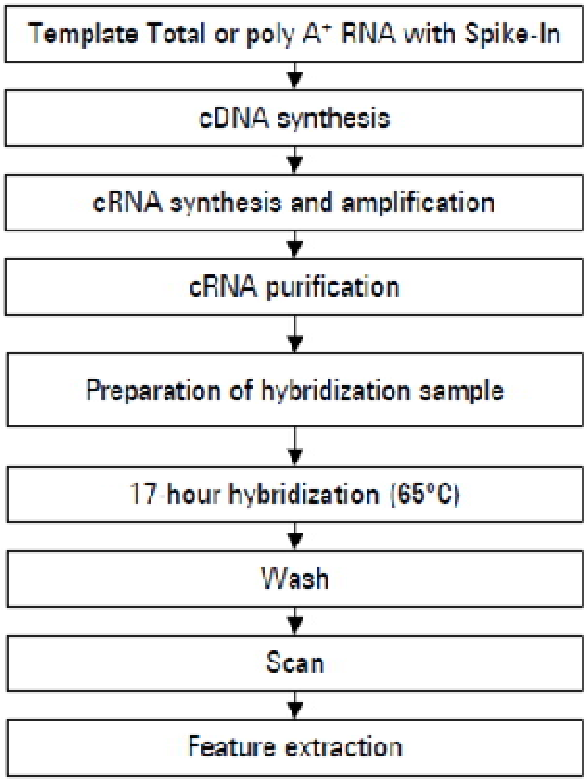


Figure 6.1: The work flow for sample preparation and array processing

Microarray Analysis

Preparation of probes, labeling and hybridization

Complementary RNA (cRNA) was generated from 1µg of total RNA using Quick-Amp Labeling kit (Agilent technologies) that directly incorporates Cy-3 labeled CTP into the cRNA (Fig 6.2). Briefly, 1 µg total RNA was mixed with T7 polymerase promoter primer and spike in mix and incubated at 65°C for 10 minutes for denaturation of template and snap-chilled on ice, before other components of the reaction were assembled. The cDNA mix was prepared by adding 5x First strand buffer, 0.1M DTT,

10mM dNTP, 400U MMLV-RT and 2.5 U Rnase inhibitor. The cDNA mix was added to the sample mix containing RNA, T7 promoter primer and diluted spike in mix. The reaction was incubated at 42°C for 2hr in water bath. The residual RNA in the reaction tube was then degraded and reaction neutralized by keeping the tube at 65 °C for 15 min and then cooled on ice for 5 min. For preparation of cRNA from the cDNA, transcription master mix was prepared by adding 4x transcription buffer, 0.1M DTT, NTP mix, 50% PEG, 2.5 U RNase inhibitor, 0.6 µl inorganic pyrophosphate, 0.8µl T7 RNA polymerase, 2.4 µl Cyanine C3- CTP and the reaction made upto 60µl with nuclease free water. The transcription mix was then added to the cDNA mix and incubated for 2 hrs at 40°C in water bath. The labeled/amplified cRNA was purified using RNeasy mini kit (Qiagen) following manufacturer`s instruction. The cRNA was quantified for yield and specific activity. Hybridization step was proceeded when the yield was >1.65 µg and the specific activity was >9.0 pmol Cy3 per µg cRNA. The array was hybridized using Gene Expression Hybridization Kit (Agilent Technologies, USA). Hybridization was carried out at 65°C for 17 hours. Three biological replicates of all hybridizations were performed to account for sample heterogeneity, and variations due to hybridization. The microarray experiments and data analysis were performed in consultation with ILife Discoveries, Manesar, Gurgaon and Genotypic Technology Pvt Ltd, Bangalore.

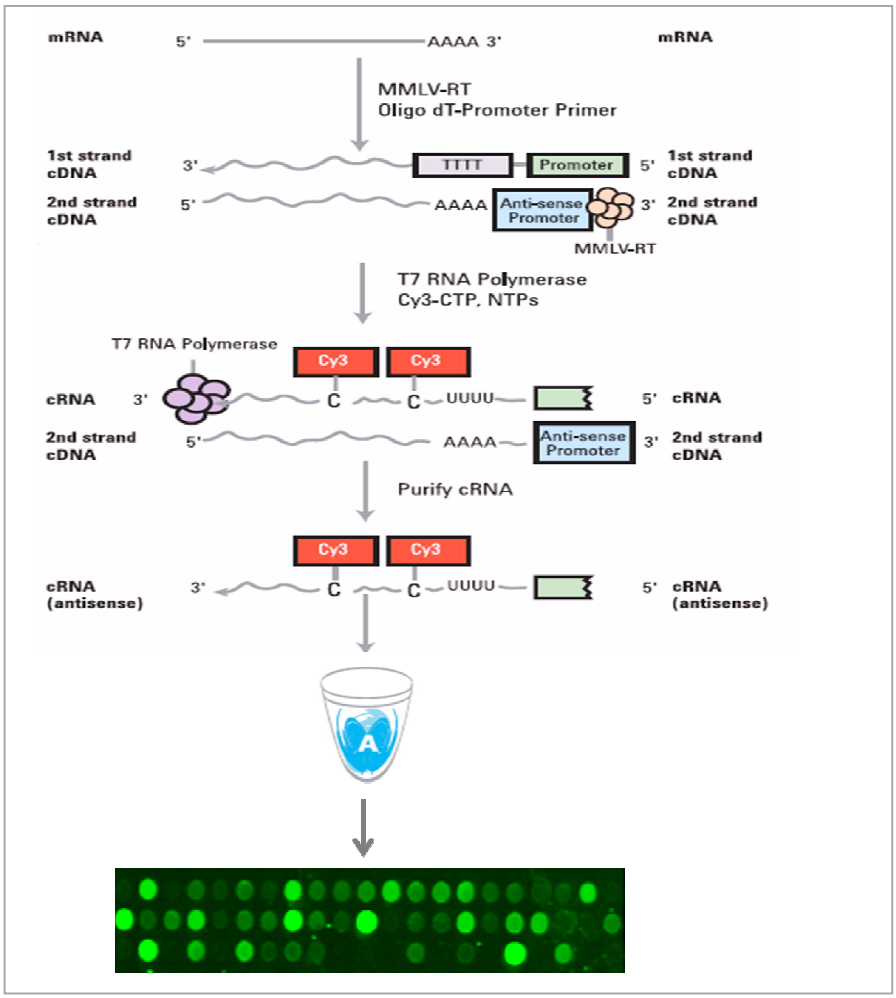


Figure 6.2: Schematic of amplified cRNA procedure. Generation of cy3 labelled cRNA from total RNA for one color microarray experiment is shown.

Description of *L. donovani* Genomic Microarray

One color microarray based gene expression profiling was carried out using *Leishmania spp.* whole genome 60mer oligonucleotide microarray slide [8X15K format].The custom designed chip shared with us by Dr Marc Ouellette at Research Centre in Infectious Diseases, Faculty of Medicine, Laval University, Quebec, Canada (Rochette et al., 2008; do Monte-Neto et al., 2011) was procured from Agilent

Technologies, USA. An overview of the steps involved in microarray based gene expression profiling is mentioned in Fig.6.3.

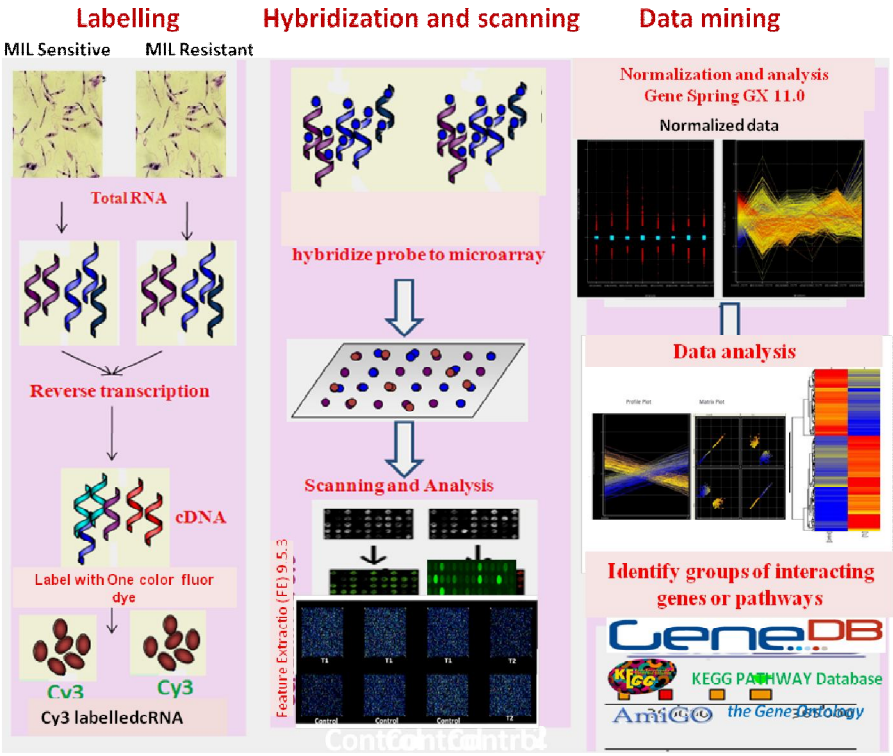


Figure 6.3: Flow diagram showing steps for microarray based gene expression profiling of Miltefosine sensitive and resistant *L.donovani*. Parasites were grown in large quantity and total RNA was isolated from the parasites. cRNA was prepared and labeled with fluorochrome Cy3 by direct incorporation using Cy3 labelled CTP. Labeled probes were hybridized with *L.donovani* genomic microarray. The image was visualized using Gene PixPro 5.0 using Feature extraction software (FE) 9.5.3 and normalized and analyzed using GeneSpring 11.0 software. The genes showing consistent and significant higher expression were BLAST analyzed in *Leishmania* GeneDB and the corresponding gene identity and ontology was determined.

Slide scanning and data processing

The hybridized arrays were washed with gene expression hybridization buffers 1 and 2 (Agilent technologies) after addition of 0.005% Triton X-102. The slides were scanned immediately in Axon GenePix 4000B scanner to minimize the impact of

environmental oxidants on signal intensities. After generating the microarray scanned images, tiff images were extracted using the Feature Extraction (FE) 9.5.3(Agilent technologies). The analysis was performed using GeneSpring GX 11.0 microarray data and pathway analysis tool. It software includes novel quantification matrices and algorithms that facilitate expression pattern analysis and gives an insight into metabolic, regulatory and disease pathways. The details of each analysis step are as mentioned below.

i. Data normalization and quality control on samples

In order to reduce variance between the arrays, quantile normalization of the samples was performed which makes the distribution of expression values of all samples in an experiment the same. Quality Control or the Sample QC was performed to decide which samples are ambiguous and which are passing the quality criteria. Based upon the QC results, the unreliable samples were removed from the analysis. The QC was done by 3D Principal Component Analysis (PCA) scores and correlation plots.

ii. Quality control on Probes

Entities were filtered based on their signal intensity values. In this step, very low signal intensity values or those that have reached saturation were removed from analysis. Identification of statistically significant differentially expressed genes was performed by t-test (Unpaired) for two groups, confidence level (p-value cut-off) < 0.05.

iii. Hierarchical Clustering

Hierarchical clustering follows an agglomerative approach, where the most similar expression profiles are joined together to form a group. The dendrogram is the most intuitive view of the results of this clustering method.

iv. Chromosome mapping

DNA microarrays data were analyzed by custom R programs to illustrate the expression profile of MIL resistant *L.donovani* by extrapolating on a chromosome map of *L.infantum*.

vii. Gene ontology and pathway analysis

The modulated genes were analyzed for their predicted protein sequences and gene ontology function using GeneDB (http://www.genedb.org/) and AmiGO analysis databases (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi). Interacting genes and pathways modulated during MIL resistance were analyzed using Gene Spring 11.0 and KEGG pathway analysis tool.

Results

RNA Quality Check: Quality Control or the RNA was performed on RNA 6000 Nano Assay Chips (Bioanalyzer). The presence of three distinct ribosomal peaks (18S, 24Sα and 24Sβ) confirmed successful RNA extraction (Fig 6.4).

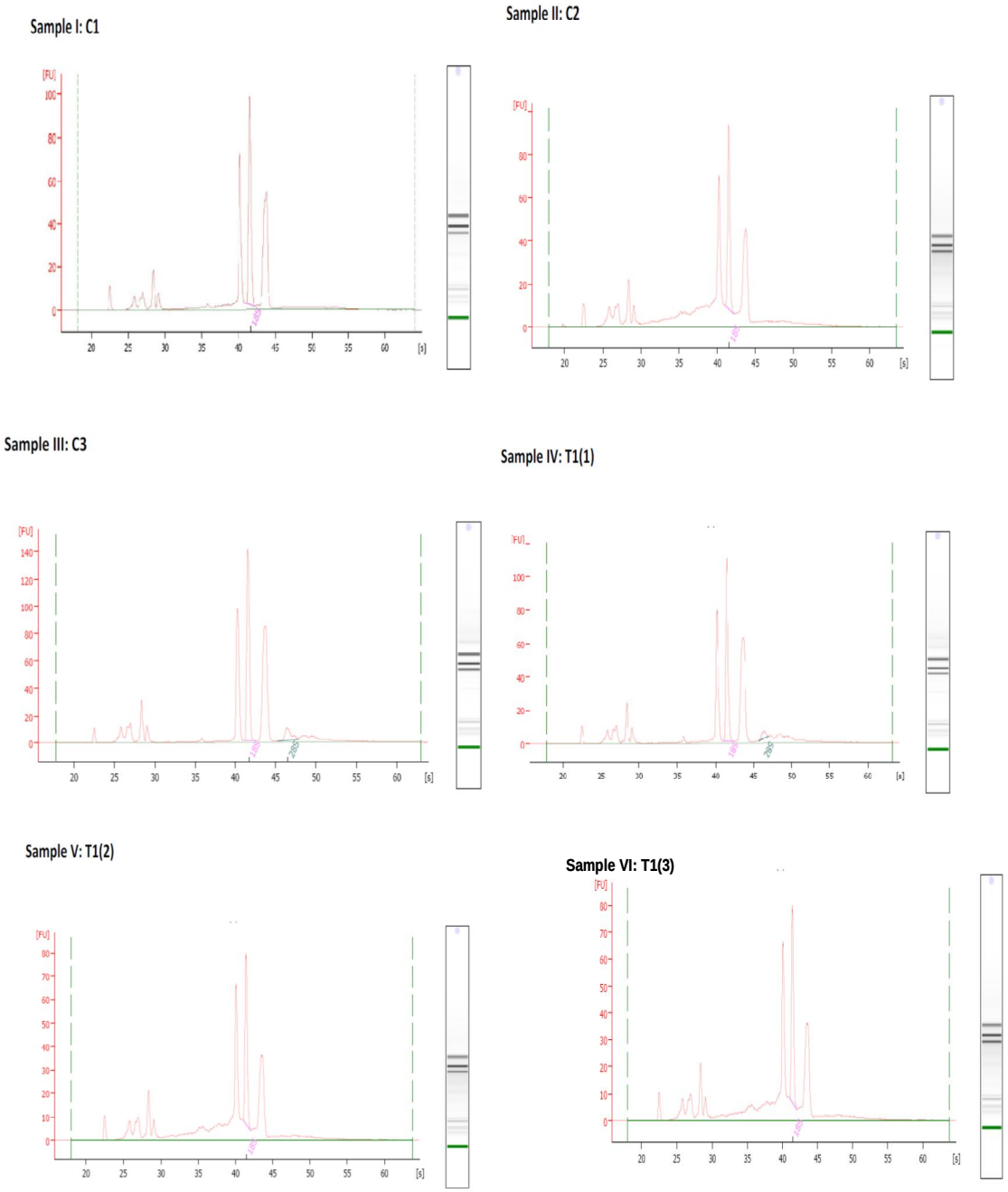


Figure 6.4: **Quality assessment of the total RNA by Bioanalyzer .** C1, C2, C3 represent control RNA from MIL sensitive; T1(1),T1 (2) and T1(3):test sample (representing MIL resistant parasite RNA).

Slide processing and hybridization

Pre-hybridization and hybridization were performed according to manufacturers' instructions. Hybridizations were performed in three biological replicates using independent RNA extractions and immediately scanned using Axon Genepix Scanner (Fig 6.5).

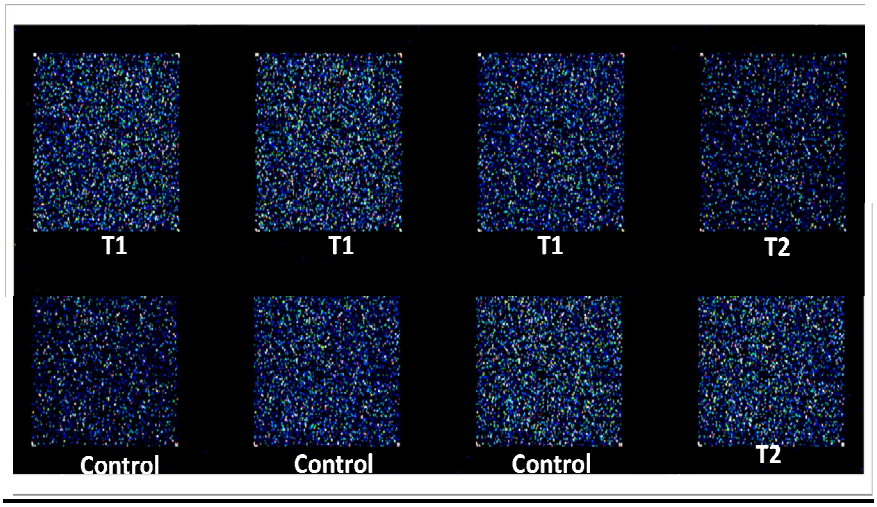
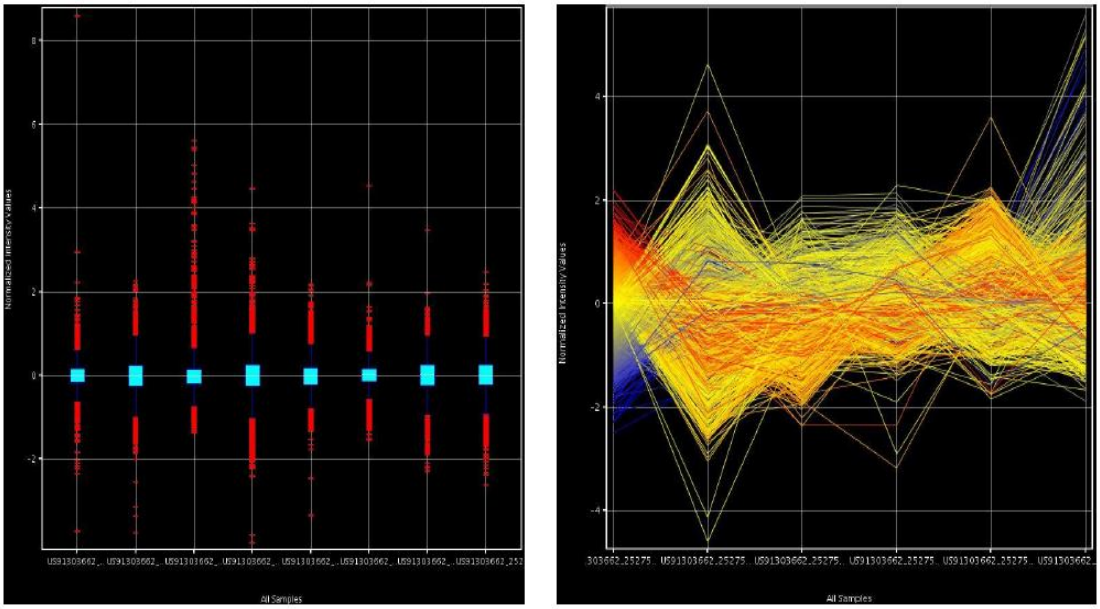


Figure 6.5: Scanned microarray slide obtained using Agilent`s feature extraction software (FE) 9.5.3. Control RNA from MIL sensitive; and MIL resistant parasite RNA (T1) were considered in the comparative analysis.

Data Extraction

Quantile normalization was performed by the following steps: The expression values of each sample was sorted in the ascending order and placed next to each other. Each column was sorted in ascending order. The mean of the sorted order across all samples was taken. Thus each row in this sorted matrix has value equal to the previous mean. The modified matrix as obtained in the previous step was rearranged to have the

same ordering as the input matrix. Normalized samples are as depicted in Fig 6.6.



T1 T1 C T2 T1 C C T2

Figure 6.6: Box Whisker and Profile plots of normalized samples. MIL resistant parasite was used as test sample (T1). The corresponding wild type sensitive parasite was used as control (C). T2 values correspond to the second test sample which has not been considered in this analysis.

Quality control on samples

Principal Component Analysis (PCA) calculates the scores and visually represents them in a 3D scatter plot. The scores were used to check data quality. The PCA components, represented in the X, Y and Z axes were numbered 1, 2, 3 according to their decreasing significance (Fig 6.7).

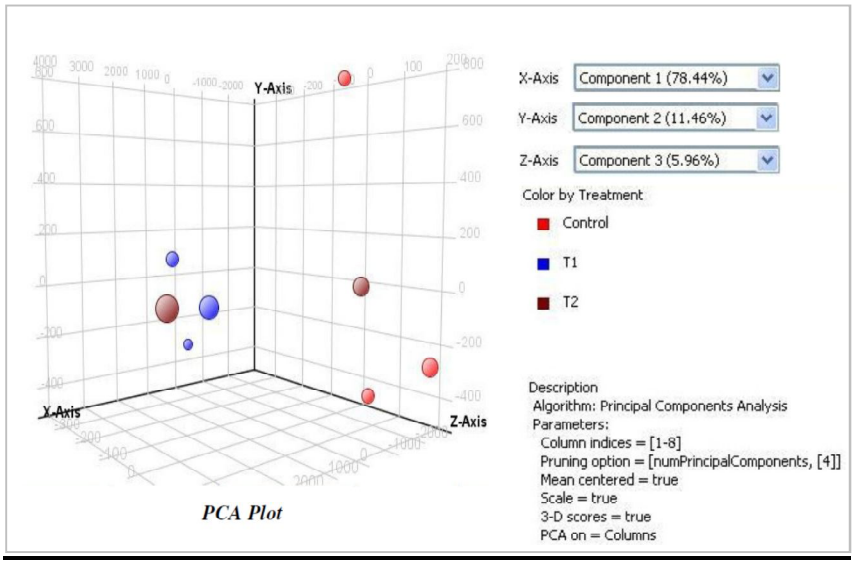


Figure 6.7: Quality control on samples used for microarray analysis (A) PCA plot of the microarray samples. PCA scores and visually represents them in a 3D scatter plot. The scores were used to check data quality. The PCA components, represented in the X, Y and Z axes were numbered 1, 2, 3 according to their decreasing significance

The combined data from replicate hybridizations were normalized and statistically validated. The data were filtered by p-value ($p < 0.05$). Out of the total 9233 entities in the array, 7963 entities passed the quality control on probes after filtering on expression. Fold change gave the absolute ratio of normalized intensities between the average intensities of the samples grouped. The entities satisfying the significance analysis were passed on for the fold change analysis. Only genes statistically significant with a difference expression ratio greater than 2.0 between wild type and MIL-R parasite were considered as differentially expressed (Fig 6.8). All microarray data is freely available on the GEO NCBI database in the MIAME format; http://www.ncbi.nlm.nih.gov/geo/ with the GEO accession number GSE 30368.

Total genes with ≤ 2 fold change in expression in MIL-R parasite vs WT: **311**

Genes showing up regulated expression in MIL resistant parasite: **184**

Genes showing down regulated expression in MIL resistant parasite: **127**

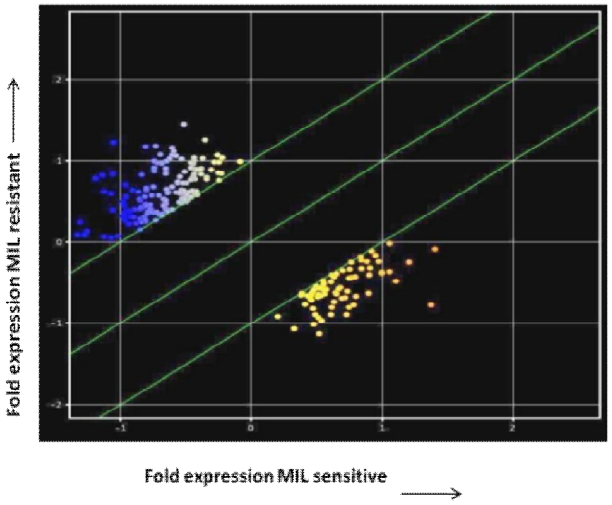


Figure 6.8: Normalized scatter plot in mRNA expression analysis. The lines on the scatter plots indicate the boundaries used for selecting genes with differential expression in MIL-R parasite. Each spot on the plots represents a specific gene. 4% of genes were outside these fold-change thresholds. Linear regression was performed.

Hierarchial clustering

Unsupervised hierarchical clustering analysis (HCA) was used to identify groups of samples that behave similarly or show similar characteristics. Hierarchical clustering algorithms build an entire tree of nested clusters out of objects in the dataset by an iterative clustering algorithm (Fig 6.9). The majority of these genes showed an average change in mRNA accumulation not exceeding 3-fold (Table 6.1)

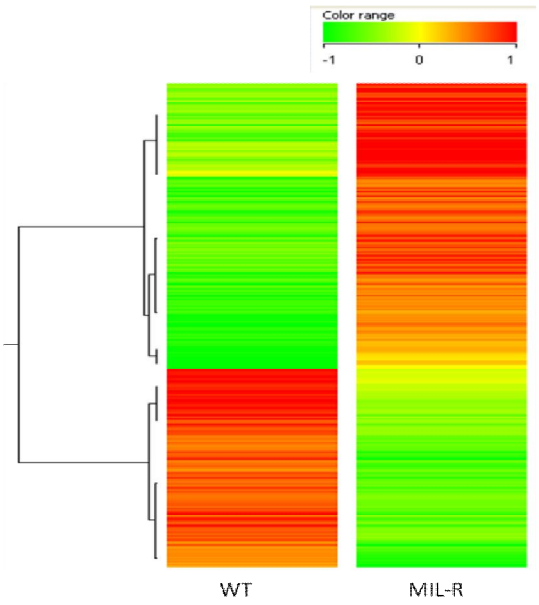


Figure 6.9: Comparison of the transcriptional responses following MIL adaptation in *L. donovani*. Heat map of log2-ratio values of genes that are differentially regulated. 2D cluster analysis was performed on genes exhibiting statistically significant variability between the two conditions as determined by Gene Spring 11.0 software. Replicate clusters (3 each) were averaged prior to cluster analysis. Cluster analysis WT- MIL sensitive *L.donovani* parasite, MIL-R- MIL resistant *L.donovani* parasite

Table 6.1: Patterns of Global mRNA expression in MIL resistant *L.donovani* parasite

mRNA upregulation (Fold increase)	MIL resistant *L.donovani*	MIL sensitive *L.donovani*
2.0 -2.5	90	89
2.5-3.0	47	19
3.0 - 3.5	23	10
3.5 - 4.5	16	9
4.5 - 6.0 fold	8	-
Total genes	184	127
% modulated genes *	2.3%	1.6%

Foot Note: The % modulated genes calculated from the total 7963 genes obtained in QC after filtering

Gene expression map of Miltefosine resistant parasite

The data generated from the microarray hybridizations can be illustrated by a chromosome map representing gene expression levels on a genomic scale (Fig 6.10). The expression ratios of every gene on a particular chromosome is represented by a color code with upregulated genes shown red feature while down regulated genes shown as green features. The expression of all chromosomes, except chromosome 1, 2 and 3 seemed to be modulated more than 2 fold in *L. donovani* MIL-R, as suggested by the map. All modulated genes from chromosomes 5, 15, 23, 26 and 29 were up regulated (represented in red) while those of chromosome 6 were mostly down regulated (represented in green). A close analysis of the normalized expression data generated by the microarray experiments confirmed that most genes located on these chromosomes were modulated by a factor ranging from 2 to 2.5 in mRNA abundance. This representation enabled the identification of one of the loci on chromosome 23 that was over expressed in MIL-R (Fig 6.10, red locus on chromosome 23). The upregulated genomic region in MIL-R comprises the genes hydrophilic acylated surface protein A (HASPA1) and small hydrophilic Endoplasmic reticulum associated protein (SHERP). Among the genes upregulated on Chromosome 29, Tob55, a member of topogenesis of mitochondrial outer membrane β-barrel proteins (TOB complex, also called the sorting and assembly machinery) plays a role in insertion of β-barrel proteins into the mitochondrial outer membrane (Paschen et al., 2003; Gentle et al., 2004). Similar gene expression map created in antimony resistance studies suggested amplification of Chromosome 23 locus that includes ABC transporter MRPA gene (Ubeda et al., 2008; Leprohon et al., 2009b, do Monte-Neto et al., 2011).

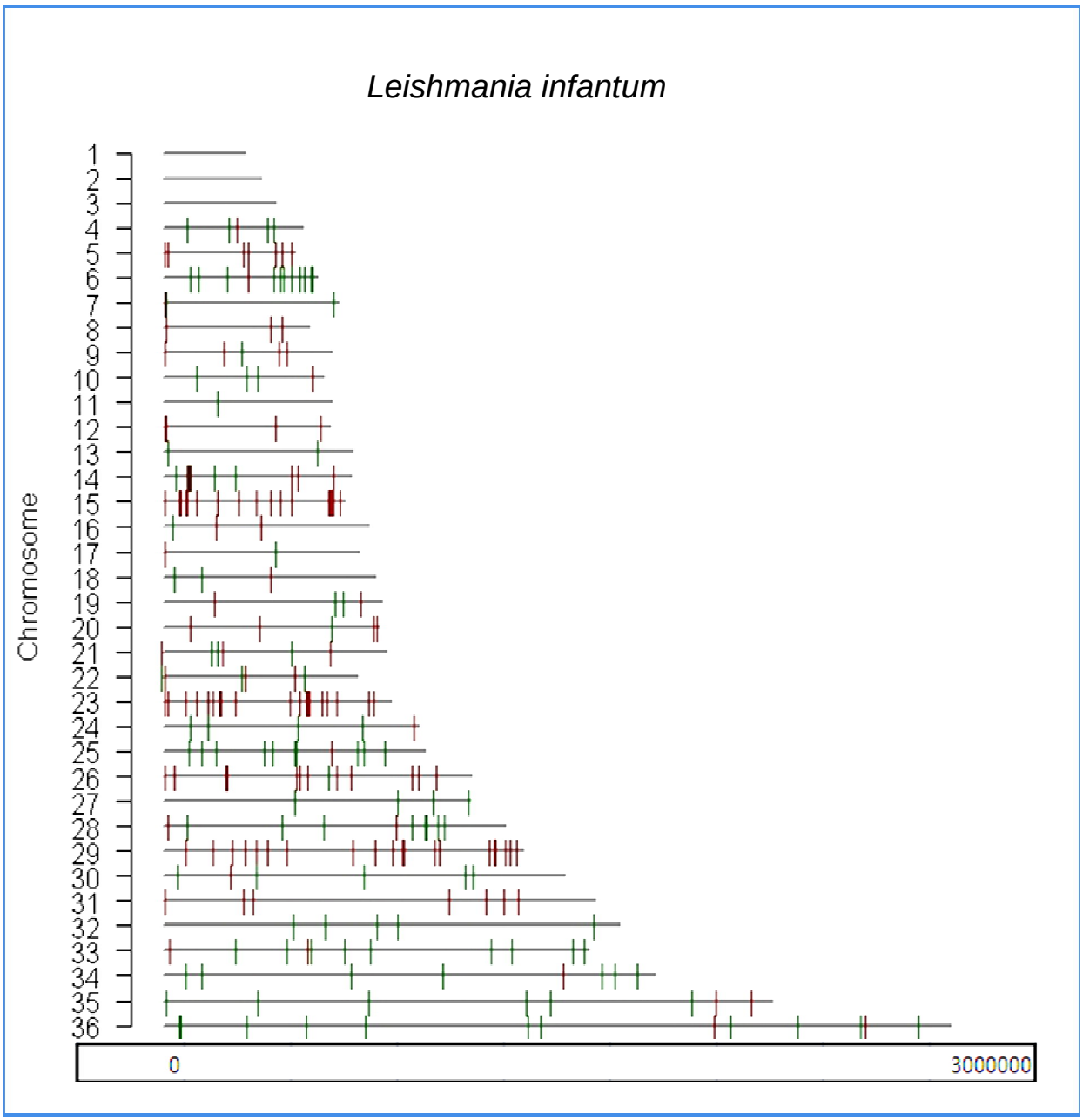


Figure 6.10: Gene expression map of *L. donovani* Miltefosine resistant/Wild Type. DNA microarray data were analyzed by custom R programs to illustrate the expression profile of *L.donovani* MIL-R/WT by extrapolating on a chromosome map of *L.infantum*. Red lines indicate up regulated genes in MIL resistant parasite, whereas green lines indicate down regulated genes. Here genes modulated 2 fold and above are plotted.

Functional categorization of the significantly altered genes

Based on their predicted protein sequence, biological/ cellular function, the genes were categorized on the basis of gene ontology using AmiGO database and GeneSpring GX11.0 software. Analysis of the genes modulated in MIL resistance showed that they belong to several categories, including molecular function, various biological processes, and cellular components (Table 6.2, Fig. 6.11). BLAST2GO and AmiGO analysis revealed that a significantly higher percentage of genes involved in metabolic process were differentially expressed in MIL-R compared to sensitive parasite. Not surprisingly, several genes involved in cellular component organization, cell cycle, cell adhesion and cell motility were upregulated specifically in MIL resistant parasites (Table 6.2).

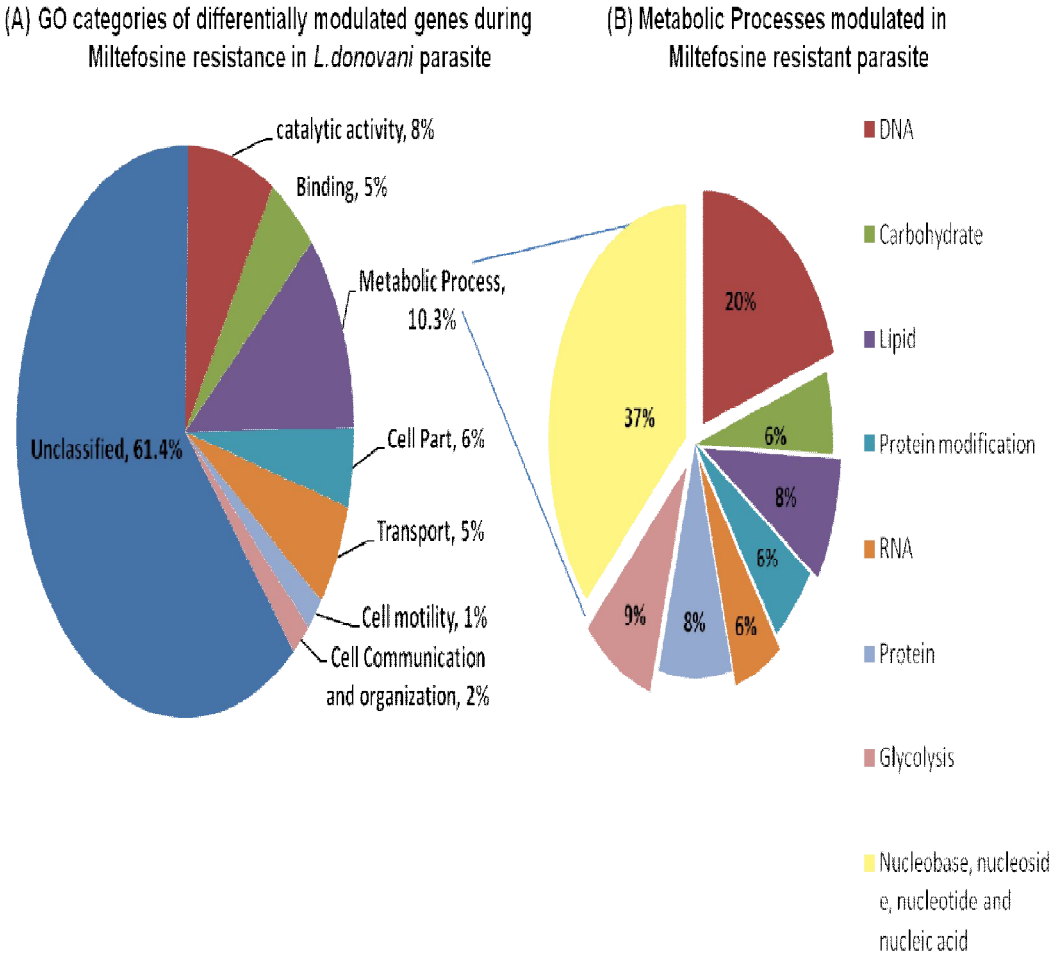


Figure 6.11: Distribution of genes differentially modulated during miltefosine resistance in *Leishmania donovani* according to gene ontology (GO) function categories (A). Overall distribution of GO categories of genes differentially expressed in MIL-R parasite suggests that genes belonging to metabolic process, transport, cell component and organization were affected. **(B)** Different metabolic processes that were modulated in MIL resistant parasite include DNA, nucleotide nucleobase, protein and lipids. Unclassified proteins include the hypothetical proteins (proteins with unknown function and not tested experimentally) and proteins with no GO category (unclassified) that have been experimentally characterized.

Table 6.2: Genes differentially modulated in Miltefosine resistant *Leishmania donovani* parasite

Gene Name	Fold change [R] vs [S]	Regulation	Product Description
Metabolic_Process	GO:0008152		
LinJ15_V3.1500	2.5	up	proliferative cell nuclear antigen (PCNA), putative
LinJ29_V3.2710	3.2	up	poly(A) polymerase, putative
LinJ23_V3.1210	3.8	up	small hydrophilic endoplasmic reticulum-associated protein (SHERP)
LinJ26_V3.1800	2.0	up	hypothetical protein, conserved
LinJ26_V3.1360	2.3	up	prefoldin-like protein
LinJ04_V3.0570	2.5	down	spermidine synthase, putative
LinJ06_V3.0370	2.5	down	glutamine synthetase, putative
LinJ10_V3.0310	2.3	down	isocitrate dehydrogenase [NADP], mitochondrial precursor, putative
LinJ11_V3.0540	2.0	down	hypothetical protein, conserved
LinJ17_V3.0930	2.1	down	hypothetical protein, conserved
LinJ21_V3.0600	2.5	down	la RNA binding protein, putative
LinJ22_V3.1110	2.2	down	ribonucleoside-diphosphate reductase small chain, putative
LinJ24_V3.0320	2.4	down	hypothetical protein, conserved
LinJ25_V3.2090	3.6	down	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase, putative
LinJ27_V3.1790	2.2	down	endo/exonuclease Mre11, putative
LinJ30_V3.0170	2.1	down	2-hydroxy-3-oxopropionate reductase, putative, pentose phosphate shunt
LinJ30_V3.1920	2.2	down	succinyl-coa:3-ketoacid-coenzyme a transferase- like protein
LinJ35_V3.3280	3.9	down	cystathione gamma lyase, putative
LinJ21_V3.1330	2.0	down	T-complex protein 1, delta subunit, putative
LinJ36_V3.0250	2.4	down	peptidyl-prolyl cis-trans isomerase, putative
LinJ13_V3.0090	4.2	down	carboxypeptidase, putative,metallo-peptidase, Clan MA(E), family 32
LinJ14_V3.0180	3.2	down	carboxypeptidase, putative,metallo-peptidase, Clan MA(E), Family M32
LinJ26_V3.1550	2.2	down	thimet oligopeptidase, putative,metallo-peptidase, Clan MA(E), Family M3
LmjF14.0180	3.2	down	carboxypeptidase, putative,metallo-peptidase, Clan MA(E), Family M32
DNA metabolic process	GO:0006259		
LinJ15_V3.1500	2.5	up	proliferative cell nuclear antigen (PCNA), putative

LinJ35_V3.4950	2.1	up	RAD51/dmc1 protein (DMC1)
LinJ21_V3.0690	2.4	down	DNA polymerase eta, putative
LinJ27_V3.1790	2.2	down	endo/exonuclease Mre11, putative
LinJ28_V3.2550	2.9	down	DNA replication licensing factor, putative
Carbohydrate Metabolic Process		**GO:0005975**	
LinJ23_V3.1060	2.2	up	beta-fructosidase-like protein,invertase-like protein,sucrose hydrolase-like protein
LinJ30_V3.0170	2.1	down	2-hydroxy-3-oxopropionate reductase, putative
Lipid Metabolic Process		**GO:0006629**	
LinJ31_V3.0870	3.2	up	lipase precursor-like protein
LinJ31_V3.2540	2.6	up	lipase, putative
LinJ14_V3.0700	2.6	down	fatty acid elongase, putative
Nucleobase, nucleoside,nucleotide and nucleic acid metabolic process		**GO:0006139**	
LinJ29_V3.2710	3.2	up	poly(A) polymerase, putative
LmjF23.1086	3.2	up	small hydrophilic endoplasmic reticulum-associated protein (sherp) (SHERP2)
LinJ11_V3.0540	2.1	down	hypothetical protein, conserved,pseudouridine synthesis
LinJ17_V3.0930	2.1	down	hypothetical protein, conserved
LinJ21_V3.0600	2.5	down	la RNA binding protein, putative
LinJ21_V3.0690	2.4	down	DNA polymerase eta, putative
LinJ22_V3.1110	2.2	down	ribonucleoside-diphosphate reductase small chain, putative
LinJ25_V3.1210	2.5	down	ATPase beta subunit, putative
LinJ27_V3.1790	2.2	down	endo/exonuclease Mre11, putative
LinJ28_V3.2290	2.6	down	A/G-specific adenine glycosylase, putative
LinJ28_V3.2450	2.2	down	DNA topoisomerase ii
LinJ28_V3.2550	2.9	down	DNA replication licensing factor, putative
LinJ30_V3.0170	2.1	down	2-hydroxy-3-oxopropionate reductase, putative
LinJ32_V3.3930	2.8	down	kinetoplast DNA-associated protein, putative
Protein metabolic process		**GO:0019538**	
LinJ04_V3.0940	2.7	down	chaperone protein DnaJ, putative
LinJ24_V3.0320	2.4	down	hypothetical protein, conserved

LinJ35_V3.3110	2.1	down	ubiquitin-activating enzyme e1, putative
RNA metabolic process		**GO:0032774**	
LinJ29_V3.2710	3.2	up	poly(A) polymerase, putative
LinJ32_V3.3930	2.8	down	kinetoplast DNA-associated protein, putative
Protein modification	**GO:0006464**		
LinJ21_V3.0010	2.5	up	phosphoglycan beta 1,3 galactosyltransferase 2 (SCG2)
LinJ35_V3.3110	2.1	down	ubiquitin-activating enzyme e1, putative
Proteolysis	**GO:0006508**		
LinJ08_V3.0950	2.1	up	cathepsin L-like protease
LinJ08_V3.0960	2.2	up	cathepsin L-like protease
LinJ19_V3.1460	2.2	down	cysteine peptidase A (CPA) (CPA
catalytic activity	**GO:0003824**		
LmjF15.1480	2.6	up	cAMP specific phosphodiesterase, putative
LinJ23_V3.0580	2.1	up	acetyl-CoA synthetase, putative
LinJ23_V3.0080	2.6	up	agmatinase-like protein
LinJ20_V3.1740	2.5	up	aminoacylase, putative,N-acyl-L-amino acid amidohydrolase, putative
LinJ25_V3.1540	2.8	up	calpain family cysteine protease-like protein
LinJ23_V3.0700	2.2	up	hypothetical protein
LmjF23.1665	2.0	up	hypothetical protein
LinJ29_V3.2880	2.7	up	hypothetical protein, conserved
LinJ15_V3.0050	3.5	up	cytochrome-b5 reductase, putative
LinJ09_V3.1120	2.2	up	DNA-directed RNA polymerase III subunit, putative
LinJ29_V3.1670	2.2	up	GTPase activator protein, putative
LinJ19_V3.1670	2.8	up	hypothetical protein, unknown function
LinJ15_V3.0170	2.8	up	protein phosphatase 2C, putative
LinJ26_V3.1800	2.0	up	hypothetical protein, conserved
LinJ10_V3.1360	3.2	up	hypothetical protein
LinJ08_V3.0060	2.8	up	phosphoglycerate mutase protein, putative
LinJ15_V3.1630	2.9	up	protein kinase, putative
LinJ22_V3.0630	3.0	up	protein kinase, putative,serine/threonine protein kinase sos2, putative
LinJ08_V3.0870	4.4	up	protein kinase, putative
LinJ31_V3.2540	2.6	up	lipase, putative

LinJ23_V3.0500	2.4	up	trypanothione synthetase, putative
LinJ15_V3.1540	3.1	up	cAMP specific phosphodiesterase
LinJ15_V3.1120	2.1	up	tryparedoxin peroxidase (TRYP)
LinJ12_v4.0050	2.0	up	hypothetical protein, conserved
LinJ36_V3.3250	2.4	down	ATP synthase, putative
LinJ28_V3.2610	3.5	down	vacuolar ATP synthase subunit b, putative
LinJ30_V3.2920	2.7	down	aldehyde dehydrogenase, putative
LinJ36_V3.1840	2.2	down	aldehyde dehydrogenase, putative
LinJ28_V3.1180	2.4	down	hypothetical protein, conserved
LinJ19_V3.1380	2.2	down	phosphatidic acid phosphatase protein-like protein
LinJ22_V3.0590	2.5	down	NADH-cytochrome b5 reductase, putative
LinJ25_V3.1860	2.6	down	hypothetical protein, conserved
LinJ10_V3.0660	2.2	down	endonuclease G, putative
LinJ07_V3.0040	2.1	down	hypothetical protein, conserved
LinJ36_V3.1420	2.2	down	Transitional endoplasmic reticulum ATPase
LinJ27_V3.0940	2.5	down	ABCA7, vesicular-fusion ATPase-like protein, putative
LinJ06_V3.0960	2.5	down	2,4-dienoyl-coa reductase-like protein
LinJ04_V3.0280	2.5	down	hypothetical protein, conserved
LinJ25_V3.1040	2.8	down	hypothetical protein, conserved
LinJ36_V3.0250	2.4	down	peptidyl-prolyl cis-trans isomerase, putative
LinJ06_V3.1070	2.6	down	kinesin, putative
LinJ34_V3.1530	2.2	down	tyrosine phosphatase isoform, putative
LinJ25_V3.1790	2.4	down	pyruvate dehydrogenase E1 beta subunit, putative
LinJ06_V3.0290	2.7	down	ribonuclease H1, putative
LmjF22.1290	2.0	down	ribonucleoside-diphosphate reductase small chain, putative
LinJ28_V3.2460	2.2	down	hypothetical protein, conserved
LinJ24_V3.0460	2.5	down	hypothetical predicted transmembrane protein
LinJ25_V3.1190	2.1	down	hypothetical protein, conserved
Binding	**GO:0005488**		
LinJ29_V3.0510	2.3	up	hypothetical protein, unknown function
LinJ15_V3.0990	2.1	up	calmodulin-like protein
LinJ29_V3.0910	2.1	up	hypothetical protein, conserved
LinJ29_V3.1530	2.9	up	clathrin coat assembly protein ap19, putative,sigma adaptin, putative

LinJ26_V3.0730	2.2	up	hypothetical protein, conserved
LinJ23_V3.0590	2.1	up	hypothetical protein
LinJ29_V3.1940	3.6	up	Tobb55,putative
LinJ16_V30930	2.2	up	flagellar calcium-binding protein, putative
LinJ18_V3.0890	2.1	up	rab7 GTP binding protein, putative (RAB7)
LinJ21_V3.1330	2.0	down	T-complex protein 1, delta subunit, putative
LinJ36_V3.0230	2.4	down	SET domain protein, putative
LinJ28_V3.1550	2.3	down	DNA polymerase kappa, putative
LinJ25_V3.1860	2.6	down	hypothetical protein, conserved
LinJ28_V3.1180	2.4	down	hypothetical protein, conserved
LinJ34_V3.3710	2.0	down	hypothetical protein, conserved
LinJ32_V3.1970	2.0	down	hypothetical protein, conserved
LinJ06_V3.1350	2.6	down	hypothetical protein, unknown function
LinJ22_V3.0002	2.3	down	hypothetical protein
LinJ36_V3.6980	2.8	down	hypothetical protein, conserved
LmjF06.1290	2.3	down	hypothetical protein, unknown function
LinJ18_V3.0300	2.1	down	hypothetical protein, conserved
LinJ04_V3.0280	2.5	down	hypothetical protein, conserved
LinJ35_V3.4630	2.5	down	hypothetical protein, conserved
LinJ24_V3.0460	2.5	down	hypothetical predicted transmembrane protein
LinJ32_V3.1180	2.1	down	mitochondrial carrier protein-like protein
LinJ34_V3.4070	2.6	down	hypothetical protein, conserved
LinJ19_V3.1380	2.1	down	phosphatidic acid phosphatase protein-like protein
LinJ35_V3.0070	2.0	down	prohibitin, putative
LinJ36_V3.5220	2.1	down	delta tubulin, putative
LinJ06_V3.1070	2.6	down	kinesin, putative
LinJ30_V3.1030	2.2	down	p22 protein precursor, putative
LinJ25_V3.1860	2.6	down	hypothetical protein, conserved
LinJ36_V3.0230	2.4	down	SET domain protein, putative
Transport	**GO:0006810**		
LinJ05_V3.0810	2.5	up	hypothetical protein, conserved
LinJ31_V3.0870	3.2306	up	lipase precursor-like protein
LinJ36_V3.5100	2.4	up	hypothetical protein, conserved

LinJ33_V3.1420	2.0	up	QA-SNARE protein putative
LinJ15_V3.0800	3.6	up	ATP-binding cassette protein subfamily A, member 7, putative (ABCA7)
LinJ29_V3.0660	2.6	up	BET1 Like protein, golgi vescicular membrane trafficking
LinJ36_V3.6490	3.0	up	ADP ribosylation factor 3
LinJ11_V3.0550	2.1	up	amino acid prermease /transporter
LinJ32_V3.2190	2.1	down	ABC transporter-like protein
LinJ25_V3.1210	2.5	down	ATPase beta subunit, putative
LinJ28_V3.2610	3.5	down	vacuolar ATP synthase subunit b, putative
LinJ36_V3.3250	2.4	down	ATP synthase, putative
LinJ32_V3.1180	2.1	down	mitochondrial carrier protein-like protein
Transporter activity	**GO:0005215**		
LinJ02_V3.0270	2.9	up	ABC 1 transporter
LinJ05_V3.1060	2.6	up	ATPase, putative
LinJ29_V3.1530	2.9	up	clathrin coat assembly protein ap19, putative,sigma adaptin, putative
LinJ31_V3.0030	2.8	up	Aquaglyceroporin, AQP1 transporetr
LinJ15_V3.0900	2.4	up	nucleotide sugar transporter, putative
LinJ32_V3.2190	2.1	down	ABC transporter-like protein
LinJ36_V3.3250	2.4	down	ATP synthase, putative
LinJ28_V3.2610	3.5	down	vacuolar ATP synthase subunit b, putative
LinJ32_V3.2190	2.1	down	ABC transporter-like protein
Cellular component organization and biogenesis GO:0016043			
LinJ25_V3.0990	2.1	down	gamma-tubulin
LinJ36_V3.5220	2.1	down	delta tubulin, putative
Cell Part	**GO:0044464**		
LinJ15_V3.0900	2.4	up	nucleotide sugar transporter, putative
LinJ06_V3.0780	2.1	up	hypothetical protein, conserved
LinJ14_V3.0330	2.0	up	hypothetical protein, unknown function
LinJ25_V3.1540	2.8	up	calpain family cysteine protease-like protein
LinJ29_V3.1670	2.3	up	GTPase activator protein, putative
LinJ23_V3.0700	2.2	up	hypothetical protein
LmjF23.1665	2.0	up	hypothetical protein
LinJ33_V3.1420	2.0	up	QA-SNARE protein putative
LinJ29_V3.1940	3.7	up	hypothetical protein, conserved

LinJ19_V3.0470	2.6	up	nuclear cap binding complex subunit CBP30, putative
LinJ26_V3.1360	2.3	up	prefoldin-like protein
LinJ05_V3.1210	2.0	up	surface antigen-like protein
LinJ13_V3.1400	2.5	down	chaperonin TCP20, putative
LinJ21_V3.1330	2.0	down	T-complex protein 1, delta subunit, putative
LinJ32_V3.2190	2.1	down	ABC transporter-like protein
LinJ14_V3.0700	2.6	down	fatty acid elongase, putative
LinJ24_V3.0460	2.5	down	hypothetical predicted transmembrane protein
LinJ32_V3.1180	2.1	down	mitochondrial carrier protein-like protein
LinJ34_V3.4070	2.6	down	hypothetical protein, conserved
LinJ19_V3.1380	2.2	down	phosphatidic acid phosphatase protein-like protein
LinJ35_V3.0070	2.0	down	prohibitin, putative
LinJ36_V3.5220	2.1	down	delta tubulin, putative
LinJ06_V3.1070	2.6	down	kinesin, putative
LinJ30_V3.1030	2.2	down	p22 protein precursor, putative
LinJ25_V3.1860	2.7	down	hypothetical protein, conserved
LinJ36_V3.0230	2.4	down	SET domain protein, putative
LinJ36_V3.3250	2.4	down	ATP synthase, putative
LinJ28_V3.2610	3.5	down	vacuolar ATP synthase subunit b, putative
Microtubule-based process		**GO:0007017**	
LinJ05_V3.0070	2.0	up	dynein light chain, putative
LinJ05_V3.0760	2.2	up	kinesin-like protein
Protein folding		**GO:0006457**	
LinJ04_V3.0940	2.7	down	chaperone protein DnaJ, putative
Rab GTPase activator activity		**GO:0005097**	
LinJ29_V3.1670	2.3	up	GTPase activator protein, putative
Structural molecule activity		**GO:0005198**	
LinJ36_V3.5220	2.1	down	delta tubulin, putative
Translation		**GO:0006412**	
LinJ24_V3.0320	2.4	down	hypothetical protein, conserved

Nucleic acid metabolism

Important differences were observed in the expression of genes involved in DNA metabolic processes particularly in nucleobase, nucleoside, nucleotide and nucleic acid metabolic process. Indeed, genes involved in DNA replication including one kDNA associated protein, were down regulated in MIL-R parasite (Table 6.2). Proliferative cell nuclear antigen (PCNA) was found up regulated during MIL resistance.

Protein metabolic process, folding and secretion

Several genes involved in protein metabolic process such as chaperone protein DnaJ and ubiquitin-activating enzyme e1 involved in proteolytic process, were down regulated during MIL resistance while cathepsin L-like proteases were up regulated. Among the genes involved in protein processing in endoplasmic reticulum, a hypothetical protein and a putative transitional endoplasmic reticulum ATPase were down regulated in MIL resistance. Genes encoding proteins involved in protein folding such as chaperonin TCP20, were among the down regulated genes in MIL resistance. Proteolyitic metallo-peptidases encoding genes including carboxy-peptidase, metallo-peptidase, and thimet oligo-peptidase were also down regulated during MIL resistance. An isoleucyl-tRNA synthetase involved in aminoacyl-tRNA biosynthesis during translation was among the down regulated genes.

Carbohydrate metabolic pathway

Beta-fructosidase-like protein, invertase-like protein, sucrose hydrolase-like protein were up regulated in MIL-R parasite while 2-hydroxy-3-oxopropionate reductase, was down regulated. Enzyme phosphoglycerate mutase, in glycolytic pathway was found

up regulated in MIL resistant parasite. Upregulated expression of gene encoding putative protein and putative acetyl-CoA synthetase was observed in MIL-R. In contrast, putative pyruvate dehydrogenase E1 beta subunit was down regulated in this pathway.

Transport and drug resistance

Interestingly several genes involved in Transport and transporter like activity were up regulated in MIL-R parasite which included ABC 1 transporter and ABCA7. Aquaglyceroporin (AQP1) transporter was up regulated in MIL-R which is contrary to SAG resistance mechanism where AQP1 has been reported to be down regulated. Golgi vesicular membrane trafficking protein was also up regulated in MIL-R *L. donovani*. One surface antigen like protein and Rab7 GTP binding protein, (RAB7) involved in endocytic pathway was also up regulated in MIL-R. The transporters with down regulated expression during MIL resistance included ATPase beta subunit, mitochondrial carrier protein-like protein.

Lipid and fatty acid metabolic process

Two genes associated with lipid metabolic process including lipase and lipase precursor like protein were up regulated during MIL resistance while gene associated with fatty acid elongase was down regulated.

Signal transduction

Several genes involved in signal transduction processes and cell cycle progression including calpain family cysteine protease-like protein, protein phosphatase 2C, phosphatidic acid phosphatase protein-like protein, protein kinases (protein kinase A and

serine/threonine protein kinase sos2) and cAMP specific phosphodiesterase were up regulated in MIL-R parasite.

Energy generation and oxidative phosphorylation

Several genes involved in oxidative phosphorylation were down regulated including ATPase beta subunit, vacuolar ATP synthase, its subunit B and 2,4-dienoyl-coA reductase-like protein encoding gene (Table 6.2). These facts suggest a decrease in respiratory electron transport chain and oxidative phosphorylation activities in MIL-R with respect to sensitive parasites.

Cytoskeleton and Motor proteins

A dynein light chain and kinesin like protein were up regulated in MIL-R while a delta -tubulin gene (d-tub) and kinesin protein were specifically down regulated. As described before, calpain family cysteine protease-like protein involved in cytoskeleton remodeling was up regulated in MIL-R.

Q-PCR for Validation of microarray experiments

10 genes were selected further based on Gene ontology categorization for validation by Q-PCR (Table 6.3). Results obtained by Q-PCR were consistent with the microarray data in all 10 cases and both methods agreed on the differential gene expression between MIL resistant and sensitive parasites (Fig. 6.12).

Table 6.3: Chosen target genes for microarray validation and their primers used for amplification

S.No:	Gene ID *L.infantum* database	Function/relevance based on GeneDB analysis	Forward and Reverse Primers (5'-3')	Product size (bp)
1	LinJ20_V3.0830	Conserved hypothetical protein1	F-CAG CAG TAG CGG TGC GTC T R-CAG GAG GTT GTT GGT GGC A	110
2	LinJ15_V3.0230	Milton protein family	F-AGG AAC GCC TTC TCA AAC CC R-TTG AGC CGC CAA AAG AGA GT	110
4	LinJ15_V3.0050	Cyt.B5 reductase	F- ACG CCG TTC TTT GGG TAC G R- TGC CTT CTG AGT CTC CCA CC	100
5	LinJ23_V3.1210	Small Hydrophyllic ER associated Proteins (SHERP)	F- GCC GCT TTT ACT TTG CCC TC R-CCG TCT GCA TCG GTG TAC TTC	100
6	LinJ09_V3.1060	ATPase binding domain , linked to ubiquitin proteasome system	F-GAC CTC AGT GCG GCA TTA AAA R-GAT CTC TGG CCA CGA CAT CC	110
7	LinJ15_V3.1540	cAMP specific phosphodiesterase	F-GTT CCT GAG GCG GTA GAG TCC R-GCA CGG CGA TAC CGT TTT AT	101
8	LinJ29_V3.1940	Hyp Protein, Mitochondrial Tansmembrane protein	F- CAA CAA GAT CCA GGA GCT GAA R-GCC GCT TAT CTT GTC CTT GA	110
9	LinJ31_V3.0870	Lipase like precursor, lipid metabolism	F-TTG GAC TTC TGG CTC ACG C R-AAG GCT GCT GTA AGC GCT G	100
10	LinJ31_V3.2540	Lipase, Putative, lipid metabolism	F-CAC AGC ACC TCG GAA GTG TTC R- GAA CAT TGC GAG GGT GCA C	121
11	LinJ13_V3.0090	MetalloCarboxypeptidase	F-ATACAGTGGACAAGTGCATCCGCA R-AGGTCTTCCGTGTTGTAGAGGCAT	128

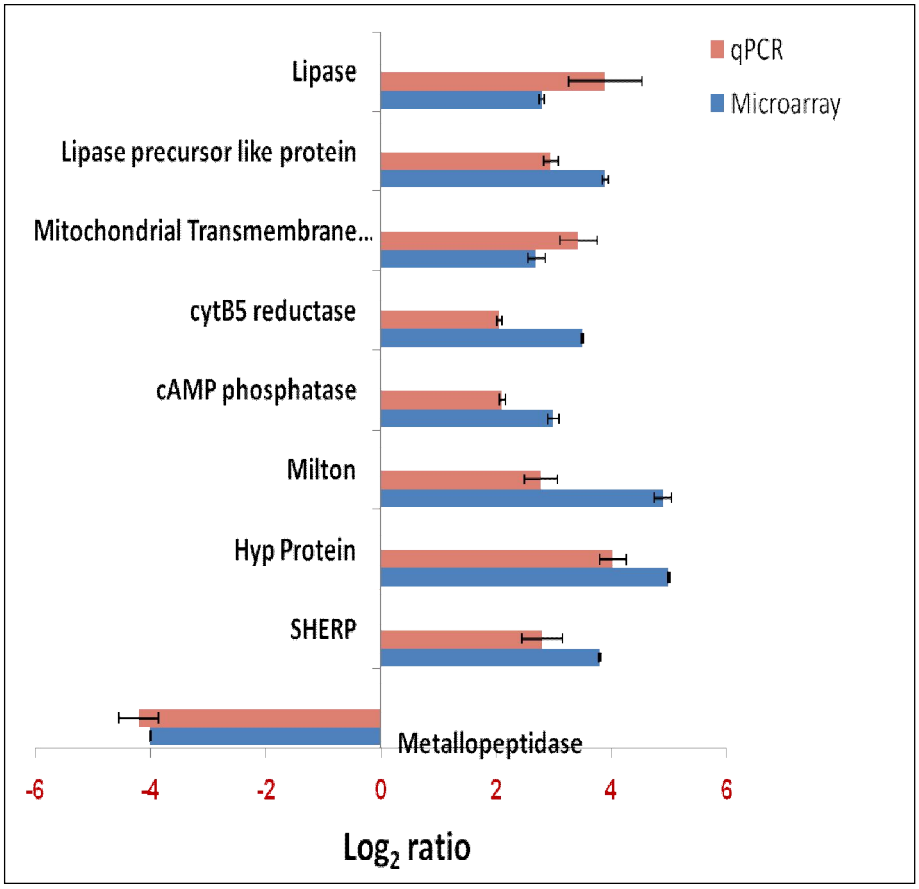


Figure 6.12: Validation of DNA microarrays expression data by quantitative real-time RT-PCR (Q-PCR). The mean log2-transformed MIL resistant/sensitive ratios of selected genes from microarrays expression data (blue bars) are compared to Q-PCR data (pink bars).

Discussion

Introduction of oral drug Miltefosine (MIL) represents an important therapeutic advance in the treatment of VL in the Indian subcontinent. However, the appearance of resistance remains a serious threat. To understand the mechanism of MIL resistance in *L. donovani*, we carried out mRNA expression profiling on full genomic DNA microarrays, a technique successfully employed to study resistance mechanisms in *Leishmania* (Ubeda et al., 2008). Mining of the *Leishmania* genome revealed the modulated expression of a number of genes that might play important role in rendering resistance to the parasite. To our knowledge, this study is the first one to report global mRNA expression modulations during MIL resistance in *Leishmania* parasite. We employed promastigote stage of the parasite for the study as there is a good correlation in the sensitivity of promastigote and amastigote forms towards MIL (Seifert et al., 2003; Kumar et al., 2009b). There is 99.9 % sequence similarity between *L. infantum* and *L. donovani* genome that justifies the use of this array.

The differential gene expression analysis in the present study suggests that the parasite adopts several strategies to counter the toxic effect of MIL (summarized in Figure 6.13), depicted by increased expression of ABC1 transporters, antioxidant defense mechanism, protein metabolism, lipid degradation and a reduced oxidative phosphorylation.

(i) Up regulated Transporters: During MIL resistance, there are numerous membrane modifications at the level of plasma membrane (as depicted by up regulated expression of ABC transporters and HASPAs) as well as mitochondria (SHERP and Tobb55) and Endoplasmic reticulum (SHERP).

ATP-binding cassette (ABC) transporters are transmembrane proteins that actively pump substrates (including metabolic products, lipids and sterols) across extra- and intracellular membranes resulting in lower substrate accumulation or a faster rate of substrate elimination (Légaré et al., 2001a). Over-expression of ABC efflux pumps P-glycoprotein (homologous to MDR1) has been associated with antimonial resistance in *Leishmania* (Légaré et al., 2001b). The involvement of an efflux pump, LMDR1/LABCB4, a P-glycoprotein-like transporter of ABC family, has been observed previously (Perez Victoria et al., 2006a). Results of the current study indicate that ABC transporters are up regulated in MIL resistance that possibly play a role in efflux of MIL in the resistant parasites. In this line, ABC protein subfamily A, ABCA7, ABC1 and ATPase were found up regulated while another ATP1 transporter like protein was down regulated in MIL resistance. In humans, these genes are known to be responsible for release of cellular cholesterol and phospholipids to apolipoproteins (Abe-Dohmae et al., 2004; Vasiliou et al., 2009). The inhibition of ABC transporter with resistance modifying agent might allow accumulation of MIL in the resistant parasite and render it sensitive. Hence one of the possible strategies to combat resistance is to circumvent drug efflux by ABC transporters to retain the drug for a longer duration in the cell.

There are evidences that phosphorylation may play an important role in ABC transporter function. Phosphorylation by protein kinase A or C has been demonstrated for several ABC transporters, including P-glycoprotein (Vanoye et al., 1999), and for ABCA1 (See et al., 2002; Martinez et al., 2003; Yamauchi et al., 2003). The transcriptome profiling in the present study, revealed up regulated expression of protein kinases that might possibly be modulating the activity of ABC transporters by

phosphorylating specific sites. Earlier reports on P-glycoprotein, an ATPase that transports drugs out of cells and confers multidrug resistance suggested that it is phosphorylated by protein kinase C (PKC) that stimulates drug transport/ drug efflux and PKC blockers rendered sensitivity to the cells (Roosbeek et al., 2004). Inhibitors of Protein kinases may also reduce MIL resistance.

SHERP is a 6.2-kDa small hydrophilic endoplasmic reticulum- associated protein that is exclusively expressed only in the metacyclic parasites (Brodin et al., 1992; Flinn & Smith, 1992; Coulson et al., 1997).It shows weak association with the ER and outer mitochondrial membrane (Knuepfer et al., 2001) and is involved in various cellular processes such as protein-import in organelles and recruitment of anionic lipids during metacyclogenesis. The up regulated expression of this gene in MIL resistant parasite as evident from this study indicates the possibility of increased parasite fitness in resistant strains.

Tob55, a member of topogenesis of mitochondrial outer membrane β-barrel proteins (TOB complex, also called the sorting and assembly machinery) plays a role in insertion of β-barrel proteins into the mitochondrial outer membrane (Paschen et al., 2003; Gentle et al., 2004). Up regulated expression of Tob55 mitochondrial outer membrane protein in the present study indicates the potential activity of MIL in modulating translocation of ions and small molecules across the mitochondrial membrane in the resistant parasites.

(ii) Reduced Protein synthesis and degradation: Genes involved in translation, protein folding and degradation were found down regulated in MIL resistant parasites. The findings suggested increased half life of proteins in MIL resistant parasite since protein

synthesis as well as degradation was down regulated. Ubiquitin-conjugating enzyme E1 gene involved in proteasomal degradation was down-regulated during MIL resistance in the present study suggesting decreased ubiquitin labelling of specific substrates for protein degradation during MIL resistance.

Prefoldin is one family of chaperone proteins found in the domains of eukarya and archaea. This protein acts in combination with other molecules as a chaperone protein and promotes protein folding in cells where there are many other competing pathways for protein folding (Martín-Benito et al., 2002). Interestingly, prefoldin transcript was up regulated in MIL resistance suggesting compensation for reduced TCP20 and DnaJ chaperons.

Papain family cysteine proteases are key factors in the pathogenesis of cancer invasion, arthritis, osteoporosis, and microbial infections. *In vitro* experiments indicated that cancer cell adaptation to increased amounts of doxorubicin over time was prevented in the presence of a cathepsin L inhibitor, suggesting that inhibition of this enzyme not only reverses but also prevents the development of drug resistance (Zheng et al., 2009). In protozoan parasites, these proteases have been known to play an important role in the infection, replication, development and metabolism (Mottram, 1998; 2004). The elevated levels of cathepsin L transcript, as inferred from the current data, suggest its role in MIL resistance. Targeting this enzyme family is therefore one strategy in the development of new chemotherapy for a number of diseases.

(iii) Altered Energy Utilization: In the present study, enzymes associated with oxidative phosphorylation pathway were down regulated in MIL-R viz ATPase beta subunit, vacuolar ATP synthase subunit B and ATP synthase. Oxidative phosphorylation is a

metabolic pathway that uses energy released by the oxidation of nutrients to produce ATP. Although oxidative phosphorylation is a vital part of metabolism, it produces reactive oxygen species such as superoxide and hydrogen peroxide, which lead to propagation of free radicals, damaging cells and contributing to disease and senescence (Cardoso et al., 2010). The enzymes carrying out this metabolic pathway are also the target of many drugs and poisons that inhibit their activities. However, when the ability of cells to generate ATP through mitochondrial oxidative phosphorylation is compromised, cells are able to adapt to alternative metabolic pathways, such as increasing glycolytic activity, to maintain their energy supply. Acetyl-CoA, formed by up regulated enzyme acetylcoA synthetase in the TCA cycle, may be being used to produce energy and electron carriers.

Lipases catalyze the hydrolysis of fats to form glycerol and fatty acids. The metabolism of fatty acids, therefore, consists of catabolic processes that generate energy and primary metabolites from fatty acids, and anabolic processes that create biologically important molecules from fatty acids and other dietary carbon sources. During MIL resistance, a putative lipase and lipase precursor-like genes were found up regulated. Fatty Acids from lipid degradation could be destined to beta oxidation and to sphingolipid biosynthesis processes. Triglycerides yield more than twice as much energy for the same mass as do carbohydrates or proteins. Secreted lipases have been implicated as virulence factors in some pathogens. It has been reported that this enzyme plays a role in acquiring lipids from the insect vector and human host to satisfy the parasite's need for energy metabolism (i.e. beta oxidation). Further, it is hypothesized that this enzyme has important functions in the synthesis of complex lipids and in the structural remodeling of

membrane lipids during parasite development. The functional activities of this parasite enzyme suggest that it could play a significant role in the pathophysiology associated with this human disease. No differential regulation of any other gene involved in phosphocholine biosynthesis was detected in MIL resistant parasites.

(iv) Antioxidant defense mechanism and tolerance to drug induced cell death: Generation of oxidants has been identified as the primary mechanism of drug induced apoptotic cell death (Moreira et al., 2011). The parasite adopts various defense mechanisms to cope with oxidative stress induced by drug by expressing antioxidant enzymes and proteins that decrease superoxide radical production. Over expression of HSP70 was found to serve as a first nonspecific stress response of *Leishmania* against antimony (Brochu et al., 2004). HSP83, identified by proteomic analysis, has been reported to provide protection against antimony induced PCD (Vergnes et al., 2007). Upregulated expression of prefoldin chaperone family may likewise be contributing in parasites` defence against MIL pressure.

A common feature of trypanosomatids is their ability to transform glutathione (GSH) into trypanothione [T(SH)2], which is the bisglutathionyl derivative of spermidine. T(SH)2 has been shown to be of pivotal importance for the vitality and virulence of trypanosomatids. Enhancement of the anti-oxidant metabolism of *Leishmania* parasites, dependent upon T(SH)2, has been implicated in drug resistance due to its central role in defence against oxidant damage, ribonucleotide metabolism and in resistance to certain drugs (Miller et al., 2000). Conversion of GSH to T(SH)2 involves enzyme trypanothione synthetase (TrS, rate limiting). T(SH)2 has been reported to play a role in antimony resistance in *Leishmania* (Oza et al., 2003; Goyeneche-Patino et al.,

2008). The up regulated expression of TrS in MIL resistance raises the possibility of its role in resistance to MIL as well. One of the principal functions of T[SH]2 within *Leishmania* is to provide reducing equivalents to facilitate the parasite's defence against oxidative stress (Fairlamb & Cerami, 1992; Flohé et al., 1999) with enzymes of the tryparedoxin peroxidase (TryP) family considered to be principally responsible for detoxification of peroxides (Flohé et al., 2003). Elevated levels of TryP, key enzyme in hydroperoxide detoxification, were observed in antimonial resistant (Wyllie et al., 2011) and arsenite resistant (Lin et al., 2005) *Leishmania* parasites thereby enhancing the antioxidant defences. Upregulated expression of TryP in MIL resistant parasite implies that enhanced antioxidant defences, through overexpression of TryP, may well be a key feature of resistance to MIL. Since exposure to MIL is known to lead to accumulation of damaging reactive oxygen species within *Leishmania* parasites, it is easy to envisage how an enhanced ability to detoxify the metabolites confers resistance in the parasite.

Prohibitin has been reported to play an important role in the events leading to *Leishmania*-host interaction (Jain et al., 2010). Its homolog has been studied in *Trypanosoma brucei* and has been reported to be upregulated upon induction of apoptosis (Welburn & Murphy, 1998). Down regulation of prohibitin in the present study possibly indicates that the MIL resistant cells are able to survive by suppressing the expression of pro-apoptotic proteins.

In conclusion, MIL resistant parasite uses alternative strategies for protein folding, degradation, transport of ion and small molecules and energy utilization to subvert the drug effects and may contribute towards understanding the mechanism by which *Leishmania* evades MIL induced death. In order to better understand these

findings, the generation of over expressing/knockout parasites for these genes could be helpful, especially for grouping genes with the same expression pattern. Such knowledge could be helpful in monitoring resistance in clinical isolates or to subsequently target major molecular interactions associated with such resistance and even to find interacting drugs able to overcome the MIL resistance phenotype and therefore increase the life span of this valuable class of drug.

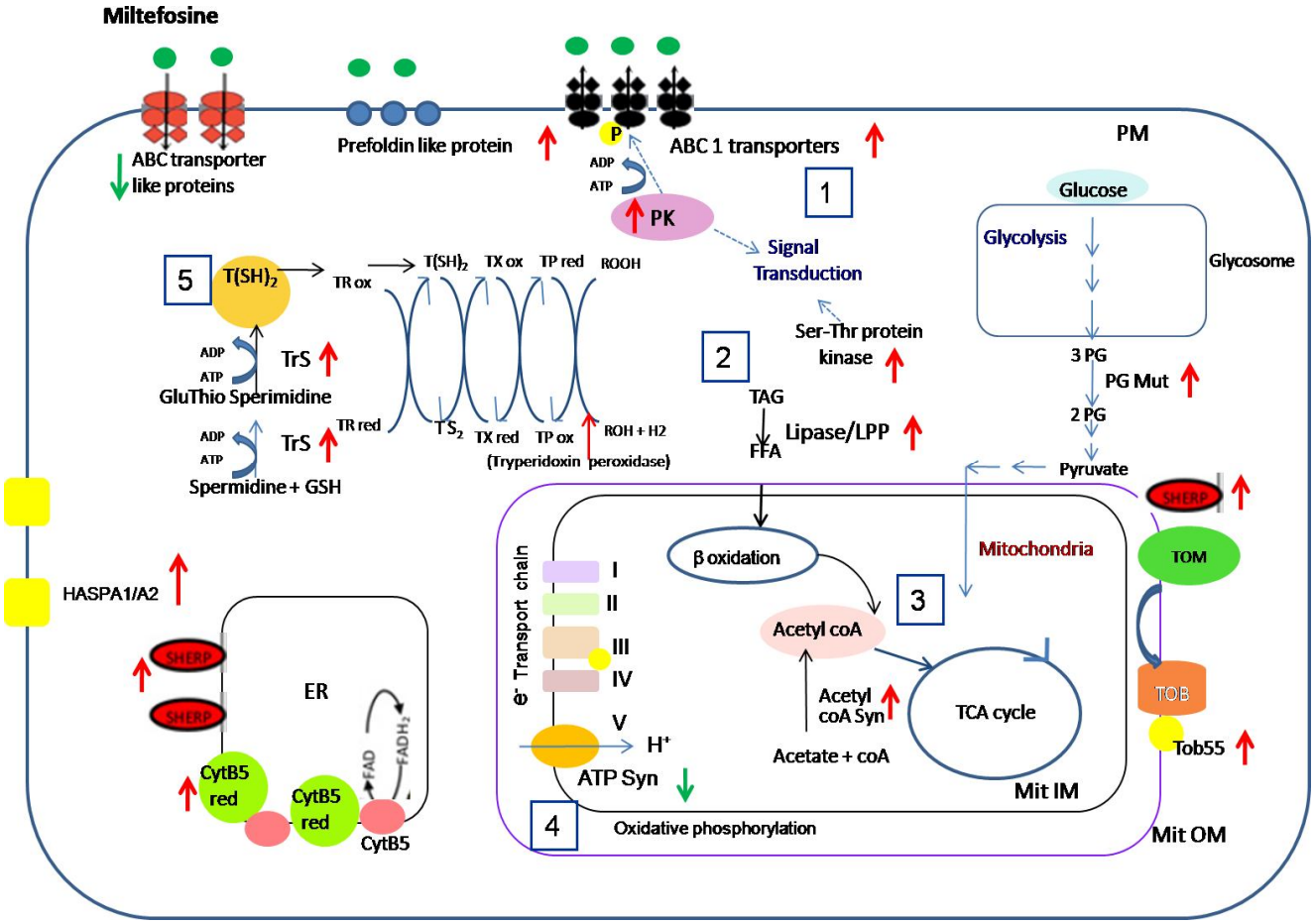


Figure 6.13: **Model depicting mechanism of Miltefosine resistance in *L. donovani*.** Genes altered in MIL-R parasite are represented. Genes marked with red arrow represent the up-regulated genes and the ones marked with green arrow represent the down-regulated genes in case of MIL-R parasite. 1, 2, 3 and 4 represents the probable mechanisms of resistance in the MIL-R isolate. **(1)** ABC 1 transporters (ABCA1 and A7) up regulated in MIL-R that lead to efflux of MIL out of the cell. Protein kinase (PK),known to phosphorylate the ABC1 transporters, stabilize the expression of these proteins on plasma membrane, possibly contributing to MIL-R. **(2)** Lipases up regulated in MIL-R are involved in fatty acid metabolism and free fatty acids (FFA) from lipid degradation could be destined to beta oxidation for energy generation as an alternate energy source **(3)** Upregulation of AcetylcoA synthetase (involved in TCA cycle) in MIL-R **(4)** Reduced oxidative phosphorylation (due to down regulated expression of ATPase beta subunit, vacuolar ATP synthase subunit B and ATP synthase) contributes to reduced reactive oxygen species generation and prevents oxidative damage in MIL-R.**(5)** Upregulation of Trypanothione [T(SH)2] biosynthesis by trypanothione synthetase (TrS) in MIL-R enhances the anti-oxidant metabolism of *Leishmania* parasites thereby contributing to MIL resistance. Up regulation of

tryparedoxin peroxidase [TP(ox)], responsible for hydroperoxide detoxification further aids in antioxidant defense in MIL-R**.**

GSH-Glutathione, [T(SH)2]-Trypanothione, (TrS)-trypanothione synthetase, TP(ox)-tryparedoxin peroxidase, CytB5-CytochromeB5, CytB5 red-CytochromeB5 reductase, ER-Endoplasmic reticulum, SHERP- Small Hydrophilic Endoplasmic Reticulum Associated Protein, PK-Protein kinsae, ATP syn- ATP synthase, PG Mut- Phospho glycerate mutase, TOM-Translocators of outer membrane, Tob- Topogenesis of mitochondrial outer membrane β-barrel proteins, Mit OM- Mitochondrial outer membrane, Mit IM- Mitochondrial inner membrane, HASPA- Hydrophilic acylated surface protein A, LPP- Lipase precursor like proteins, FFA-free Fatty acid, TAG-Tri acyl glycerol.

Conclusions and future scope of work

Conclusions and future scope of work

The surveillance of drug susceptibility in the prevailing *Leishmania donovani* parasite population is a high priority issue due to emergence of resistance to traditional antimony therapy and limited available alternatives for VL treatment. With recent introduction of MIL therapy for VL, and new drugs paromomycin and sitamaquine likely to be introduced soon, our study provides the baseline data on the intrinsic sensitivity of the prevailing *L. donovani* parasite population, yet unexposed to these drugs, that will contribute in formulating the right and more effective treatment strategies to increase the lifespan and efficacy of these drugs. In the present study, sensitivity of the clinical isolates of *L. donovani* towards miltefosine (MIL) and AmphotericinB (AmB), the drugs currently in use for VL treatment, was evaluated at both promastigote and intracellular amastigote stage. The field isolates (n=19) exhibited variable susceptibility to these drugs with ED_{50} at amastigote stage ranging from 0.48 to 2.32 µg/ml for MIL and 0.17 to 0.77µg/ml for AmB. Isolates from high SAG resistance zone (n=12) exhibited significantly lower susceptibility for both the drugs in comparison to those from low SAG resistance zone (n=7) indicating the possible risk of development of resistance to these drugs in this area. A strong correlation was observed for MIL susceptibility between the two stages of parasites i.e. amastigotes and promastigotes, that demonstrated the utility of promastigote assay as a simplified biological tool for MIL susceptibility testing in field. With establishment of resazurin assay as a simplified tool, surveillance of MIL susceptibility can be done in field in clinical isolates from MIL treated patients.

Paromomycin (PMM) and sitamaquine (SIT) are the two new drugs likely to be introduced for VL therapy in field. Utmost care will need to be taken to prevent the development of resistance to these drugs. We determined the baseline data on natural *in vitro* susceptibility in the prevailing field isolates previously unexposed to these drugs. Field isolates exhibited a variable sensitivity to PMM and SIT and the susceptibility at the two parasite stages did not correlate for either drug suggesting that amastigotes are suitable models for monitoring parasite susceptibilities towards these drugs. Isolates from high SAG resistance zone exhibited significantly lower susceptibility for SIT in comparison to those from low SAG resistance zone, while isolates from different zones showed similar susceptibility to PMM. Further, we observed that nitric oxide (NO) release was promoted in *L. donovani* infected macrophages upon treatment with PMM/SIT. NO inhibitor significantly compromised the amastigote killing by SIT but not by PMM indicating that NO is involved in antileishmanial action of SIT but not PMM. In conclusion, SAG resistant/sensitive VL isolates were susceptible to both PMM and SIT. PMM, exhibiting higher efficacy towards SAG resistant parasites and having a distinct mechanism of action, appears a promising drug for combination therapy. Additionally, the study identified extracts from marine organisms showing potent antileishmanial activity, of which the highest efficacy was exhibited by sponge *Haliclona.*

We generated MIL resistant parasite by stepwise increase in drug pressure upto 30 µg/ml MIL and characterized it. The resistant lines showed up to 18 fold lower susceptibility to MIL compared to WT parasite at promastigote stage. The resistance induced at the promastigote stage was evident at the amastigote stage where MIL-R parasite exhibited about 7 fold lower susceptibility than WT counterpart. The infectivity

was retained in MIL-R strain suggesting that in the field, resistant parasites fit to establish infections, could lead to rapid transmission of a MIL-resistant population and put the lifespan of this important drug at risk. The mRNA expression of MIL transporters, LdMT and LdRos3 (reported markers of experimental MIL resistance) was markedly reduced in experimental induced MIL-R strain but not in field isolates. The expression levels of these two genes did not correlate with the *in vitro* MIL sensitivity of field isolates. The data with intermediate stages of MIL-R parasite showed that alteration in expression was evident only in highly resistant parasites (ED_{50} values above 19 µg/ml for LdMT and above 25 µg/ml for LdRos3).The ED_{50} of the parasites in field was much below this concentration which explained why prevailing field isolates did not show an altered expression of these genes. Therefore, LdMT/LdRos3, implicated in experimental resistance to MIL, did not appear to be suitable markers to monitor drug susceptibility in field isolates. Thus, our data indicates that mechanism for MIL resistance in field may involve genes other than LdMT and LdRos3.

In an effort to understand the mechanisms/related biological pathways that contribute to MIL resistance in the parasite and to identify potential biomarkers of MIL resistance, we compared the global gene expression patterns of MIL resistant and sensitive *L. donovani* parasite by microarray analysis using whole genome *Leishmania* oligonucleotide array comprising total of 9233 genes, followed by validation using Real time PCR. Analysis resulted in identification of 311 genes representing ~ 4% of the total *L. infantum* genome that were differentially expressed (>2 fold modulated) in MIL resistant parasite. In depth analysis by bioinformatic tools revealed altered expression of genes belonging to several metabolic pathways, catalytic activity, transporters and cell

organization & biogenesis. The expression of several genes related to Trypanothione metabolism (trypanothione synthetase, tryperidoxin peroxidase) and ABC 1 mediated drug efflux were prominent among those found preferentially up regulated in the MIL resistant parasite while oxidative phosphorylation was down regulated. Overall, the study gives an insight into the mode of action and possible mechanism of resistance to MIL and identifies important molecules that may lead to potential biomarkers of MIL resistance.

Future scope of work

The well characterized MIL-R parasite generated in the current study can be subjected to whole genome sequencing or proteomic/metabolomic analysis for an in depth understanding of the resistance mechanism. Comparative analysis of genomic/proteomic approach will provide a better understanding of the mechanism of MIL resistance. It will be of interest to study the effect of resistance on the fitness of the MIL-R parasite (in terms of its response to oxidative and nitrosative stress by host cells). In addition, modulations in host responses upon infection with MIL resistant parasite will provide a clearer understanding of involvement of host factors in MIL resistance. In our study, we have identified an array of MIL resistance modulated genes and pathways in the parasite such as trypanothione and tryperidoxin metabolism that may be explored functionally for their role in MIL resistance. The other major genes of interest identified in microarray included a hypothetical protein, characterized in *Trypanosoma brucei* as Tob55, a mitochontrial transmembrane protein responsible for protein trafficking in the mitochondria and lipase precursor like protein involved in lipid metabolism. Studies towards cloning and characterization of these genes for their role in resistance will help to understand the basis of MIL resistance in *Leishmania*. Further, evaluation of key molecules identified in the study in the clinical isolates from MIL treated patients will

help to provide important leads for molecular biomarkers of MIL resistance which is the need of the hour in field. Marine products offer an unprecedented avenue for development of safe and efficacious drugs and it will be interesting to identify the active components as alternative or possibly synergistic drugs for VL therapy.

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Appendix

Appendix

Preparation of reagents

10X MOPS

0.2M MOPS pH 7.0, 20mM Sodium acetate, 10mM EDTA pH 8.0

Filter sterilized with 0.45㎜ filter.

DEPC water

0.1% diethylpyrocarbonate was added to 1ltr double distilled water in a fume hood and mixed well. After incubating it for 1hr at 37℃ it was autoclaved.

20X SSC

175.3gm of NaCl, 88.2gm of Sodium citrate were dissolved in 800ml distilled water. pH was set to 7.0 with HCl. Volume was adjusted to 1ltr, aliquots prepared and the solution was sterilized by autoclaving.

Stock solution of commonly used reagents

1M Tris

121.1gm of Tris base was dissolved in 800ml of dw and pH set (6.8, 7.4, 8.0) with concentrated HCl. Volume was made up to 1liter and autoclaved.

0.5M EDTA

186.1gm of disodium EDTA.-$2H_2O$ was added in 800ml of dw, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to1liter and autoclaved.

3M sodium acetate

204.5gm of $C_2H_3O_2Na$. $3H_2O$ was dissolved in 400ml of dw, pH set to 5.3 with glacial acetic acid, volume made up to 500 ml and autoclaved.

10% SDS

10gm of electrophoresis grade SDS was dissolved in 70ml of dw, heated at 60℃ to dissolve and the volume made up to 100ml.

Ethidium Bromide (10 mg/ml)

10mg of ethidium bromide was dissolved in 1ml dw, stored in a opaque bottle.

30% Acrylamide Stock

29.2gm of acrylamide and 0.8gm of bis-acrylamide were dissolved in 50ml of ddH_2O. Volume was made up to 100ml, the solution filtered through Whatman no. 1 paper, degassed and stored in an opaque bottle.

Calcium Chloride (0.1 M)

1.47gm of $CaCl_2.2H_2O$ was dissolved in 100ml of ddH_2O and sterilized by autoclaving.

IPTG (1M)

238mg of IPTG was dissolved in 1ml of dw, filter sterilized and stored at -20℃ in 50ml aliquots.

Coupling Buffer (0.2 M)

0.42 gm of $NaHCO_3$ was dissolved in 40 ml, pH was set to 9.0 and volume made upto 50 ml. Sterilized using 0. 45µm filter

10 X TAE buffer (Tris acetate, EDTA)

4.84gm of Tris base in 80ml of dw was dissolved and 1.2ml of glacial acetic acid and 2ml of 0.5 EDTA pH 8.0 were added. Final volume was made up to 100ml.

10X TBE buffers (Tris borate, EDTA)

8gm of Tris base, 55 gm of boric acid and 9.3gm $Na_2EDTA.H_2O$ were dissolved in 700ml dw and the final volume made up to 1 liter.

Phosphate Buffer Saline (PBS)

8gm of NaCl, 2gm of KCl, 1.44gm of Na_2HPO_4 and 0.2gm of KH_2PO_4 were dissolved in 800ml of dw. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15lb/ sq.in for 20 minutes and stored at room temperature.

DNA loading dye (6X)

0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml of glycerol were dissolved and volume set to 100 by autoclaved dw.

Publications and Presentations

Publications and Presentations

Publications

1. Bhandari V*, **Kulshrestha A***, Deep DK, Prajapati VK , Ramesh V, Sundar S, Dujardin JC and Salotra P (2011) Surveillance of drug susceptibility in Leishmania donovani clinical isolates following Miltefosine treatment in cases of Visceral Leishmaniasis and Post kala-azar dermal Leishmaniasis **PLos Neg Trop Dis** (in press) [* **Authors contributed equally**].
2. Kumar D, Singh R, Bhandari V, **Kulshrestha A**, Negi NS, Salotra P (2012) Biomarkers of antimony resistance: need for expression analysis of multiple genes to distinguish resistance phenotype in clinical isolates of Leishmania donovani. **Parasitol Res.** 2012 Feb 3**.** (Epub ahead of print)
3. Srividya G*, **Kulshrestha A***, Singh R, Salotra P (2011) Diagnosis of Visceral Leishmaniasis: developments over the last decade. **Parasitol Res.** 110(3):1065-78. **[* Authors contributed equally]**
4. **Kulshrestha A**, Singh R, Kumar D, Negi NS, Salotra P (2011). Antimony-resistant clinical isolates of Leishmania donovani are susceptible to paromomycin and sitamaquine. **Antimicrob Agents Chemother.** 55(6): 2916-21.
5. Kumar D, **Kulshrestha A**, Singh R, Salotra P. (2009)**.** *In vitro* susceptibility of field isolates of *Leishmania donovani* to Miltefosine and Amphotericin B: correlation with SAG susceptibility and implications in the endemic area. **Antimicrob Agents Chemother.** 53:835-8.
6. **Kulshrestha A**, Sharma V, Singh R, Salotra P. Genome-wide gene expression profiling for identification of Miltefosine resistance associated genes in *Leishmania donovani*. (Communicated).
7. **Kulshrestha A**, Bhandari V, Mukhopadhyay R, Ramesh V, Sundar S, Maes L, Dujardin JC, Roy S, Salotra P. Concordance between inter-stage miltefosine susceptibility of *Leishmania donovani* isolates: utility of promastigote assay as a simplified biological tool. (Communicated).

8. **Kulshrestha A**, Padmakumar K, Kumar D, Singh R, Salotra P. *In vitro* antileishmanial effect of methanolic extracts of marine sponges, mollusks and ascidians against *Leishmania donovani* (Manuscript under preparation).

Chapter in Book

1. Singh R, **Kulshrestha A**, Salotra P (2011). Research in diagnostic tools: the past, present and future. Chapter In "Kala azar- Emerging perspectives and prospects in South Asia", Ed. H.P Thakur, Mittal publishers India, PP 155-189.

Presentation/Abstracts in National/International conferences

1. **Arpita Kulshrestha**, Vanila Sharma, Ruchi Singh, Narendra Singh Negi, Poonam Salotra. Transcriptome profiling for identification and characterization of miltefosine resistance associated genes in *Leishmania donovani.* At MICROCON-2011 conference organized by Indian association of Medical Microbiologists held at BHU, Varanasi, from 23rd to 26th Nov, 2011.P-230

2. **Arpita Kulshrestha**, Lonchamp J., Inocêncio da Luz R., Bhandari V., Sundar S., Rijal S., Dujardin J.C. Maes L., Carter K.C., Coombs G. and Salotra P. Natural susceptibility of Leishmania donovani isolates from Indian subcontinent towards Miltefosine and impact of SSG resistance background on development of Miltefosine resistance. In "Neglected Protozoan Diseases Conference" held at Pasteur Institute, Paris, France from September 22nd -23rd 2010.

3. Vasundhra Bhandari, **Arpita Kulshrestha**, Dhiraj Kumar and Poonam Salotra. Drug susceptibility of Indian field isolates of L.donovani and development of experimental resistance towards Miltefosine and Paromomycin. In "Neglected Protozoan Diseases Conference" on held atPsteur Institute , Paris, France from September 22nd -23rd 2010.

4. **Arpita Kulshrestha**, Ruchi Singh, Dhiraj Kumar, Poonam Salotra .Susceptibility of Indian *Leishmania donovani* isolates towards paromomycin and Sitamaquine: new treatment options for Visceral Leishmaniasis. In National Conference on "Emerging Trends in Life Science Research" held at BITS, Pilani, India from March 6 -7, 2009. P 36.

5. **Arpita Kulshrestha**, Ruchi Singh, Dhiraj Kumar, Poonam Salotra. Susceptibility of *Leishmania donovani* clinical isolates from India towards paromomycin and

sitamaquine .In 4th World *Leishmania* Congress (WL4), held in Lucknow, India, from Feb3-7, 2009.P 162.

6. Dhiraj Kumar, **Arpita Kulshrestha**, Ruchi Singh, Poonam Salotra. *In vitro* susceptibility of Indian Kala-azar isolates to Miltefosine and expression analysis of markers for drug resistance. In 9th Sir Dorabji Tata Symposium on "Antimicrobial Resistance", held at Bangalore, India, March 10-11, 2008.P 73.

7. P. Salotra, K. Padamkumar, Dhiraj Kumar, **A. Kulshrestha,** R. Singh. Challenges in the treatment of Visceral Leishmaniasis: Potential of marine natural products as alternative drug candidates. In Keystone symposium on "Drugs against protozoan parasites", held at. California, USA, Jan28- Feb1, 2007, P 52.

8. P. Salotra, K. Padamkumar, Dhiraj Kumar, **A. Kulshrestha**, R. Singh. Potential of marine natural products as alternative drug candidates. In National Symposium on "Current trends in the development of herbal drugs" and 27th Annual conference of Indian Association of Biomedical Scientists (IABMS), Trivandrum, Kerala, Nov25-27, 2006. P- 79.

Biographies

Biography

Name Arpita Kulshrestha

Date of Birth 26 Dec' 1982

Educational qualification

Examination Passed	Board/University	Percentage	Year of passing
Secondary school Examination	CBSE	82.4	1998
Intermediate	CBSE	72.1	2000
B.Sc (General)	Delhi University,	72.3	2003
M.Sc Biotechnology	Jiwaji University, Gwalior	74.8	2005

Academic Achievements

1. Member of the Organizing Committee of the "Hands on Training in Molecular Biology Techniques" for Post graduate/MBBS students held at National Institute of Pathology (ICMR), New Delhi from March 1st -4th ,2011.
2. Was among the 22 participants selected all over the world for the **Wellcome Trust Advance Course**, organized by Wellcome Trust Sanger Institute, UK and held at ICGEB, New Delhi between 16th to 21st Dec' 2010.
3. Awarded **Senior Research Fellowship** by Council of Scientific and Industrial Research (CSIR), India (March, 2008).
4. Qualified Joint **CSIR-UGC** National examination leading to award of Junior research fellowship to pursue research (December 2004).
5. Qualified Joint **CSIR-UGC** National examination for Junior Reasearch fellowship (June 2004).
6. Secured 4th rank in All India entrance exam conducted by Jiwaji University, Gwalior, Madhya Pradesh, India for admission to post graduation in Biotechnology (2003).
7. Topper (1st, 2nd, 3rd year) of B.Sc at Shivaji College, University of Delhi.

Conferences Abstracts and presentations

- Attended National 'MICROCON-2011'conference organized by Indian Association of Medical Microbiologists, held at BHU, Varanasi from 23rd to 26th Nov, 2011.
- Attended Wellcome Trust Advance course by Wellcome Trust Sanger Institute, UK, held at ICGEB, New Delhi from 16th to 21st Dec, 2010.
- Underwent short term training on "Simplified tools for drug sensitivity studies in Leishmaniasis", at LMPH, University of Antwerp, Belgium, from 27th April to 8th May, 2009.
- Attended 4th World Congress on Leishmaniasis (WL4) held during 3rd to 7th Feb'09 at Lucknow, India.
- Attended National Conference on Emerging Trends in Life Sciences Research held at BITS, Pilani, Rajasthan, India from 6th to 7th Mar'09.
- Attended 33rd Indian Immunology society conference held at AIIMS, New Delhi, India from 28th to 31th January 2007.
- Attended 21st annual conference of Indian Association of Pathologists and Microbiologists, Delhi Chapter organized by Institute of Pathology (ICMR) and Safdarjung Hospital and V.M.M. College held on 16 April 2006.

Work Experience

- Worked as CSIR-JRF from March 2006 to February 2008 towards my PhD problem "Studies on drug sensitivity in *Leishmania donovani* field isolates and differential gene expression analysis in Miltefosine resistant parasites" under the Supervision of Dr. Poonam Salotra, Deputy Director, National Institute of Pathology (ICMR), New Delhi.
- Worked as CSIR-SRF from March 2008 to February 2010 towards my PhD problem under the Supervision of Dr. Poonam Salotra, Deputy Director, National Institute of Pathology (ICMR), New Delhi.
- Working as SRF in European Commission Funded Project "KALADRUG-R" from March 2010 till date under the Supervision of Dr. Poonam Salotra, Deputy Director, National Institute of Pathology (ICMR), New Delhi.

- Underwent short term project training on the project entitled " Upregulation of human Cathepsin-L expression in hypoxia" under the guidance of Prof S.S. Chauhhan at Department of Biochemistry, All India Institute of Medical Sciences (A.I.I.M.S) from 1st Feb to 29th June, 2005

Brief Biography of the Supervisor

Personal Particulars

- Name Poonam Salotra
- Designation : Deputy Director
- Place of work : Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi – 110 029, India.
- Tel. No: 26198402, 26166124
- Fax No: 26166124
- E. mail: salotra@vsnl.com, salotrap@icmr.org.in

Academic Qualifications

- B.Sc. Hons (Chemistry) from Delhi University in 1974.
- M.Sc. (Biochemistry) from P.G.I., Chandigarh in 1976.
- Ph.D. from V.P. Chest Institute, Delhi University in 1980.

Professional Appointments:

- Postdoctoral Fellow at Roche Institute of Molecular Biology, Nutley, New Jersey, U.S.A. (1980).
- Research Officer at Tuberculosis Research Centre, Madras (1982).
- Principal Investigator under Young Scientists scheme of Department of Science & Technology at Centre for Biotechnology, J NU, New Delhi (1991).
- Pool Research Officer (CSIR) at Centre for Biotechnology, JNU, New Delhi (1993).
- Senior Research Officer at Institute of Pathology (ICMR), New Delhi (1996).
- Assistant Director at Institute of Pathology (ICMR), New Delhi (2000).
- Deputy Director at Institute of Pathology (ICMR), New Delhi (2005 till date)

Research Interests:

- Worked mainly on the molecular basis of pathogenesis of infectious diseases such as Kala-azar, Anthrax, Tuberculosis and Cholera. Currently working on development of diagnostic tests and attenuated vaccines for kala-azar, mechanism of drug resistance in Indian kala-azar, and characterization of immune responses in patients of kala-azar and post kala-azar dermal leishmaniasis.

Awards/Honours

1. Elected Fellow of the Indian National Science Academy, in the year 2011.
2. Member of the WHO expert committee meeting on "Control of Leishmaniaisis" in 2010.
3. Elected Fellow of the National Academy of Sciences, India, in the year 2008.
4. Basanti Devi Amir Chand Award conferred by ICMR in 2007.
5. Prof. BK Aikat Award conferred by Indian Council of Medical Research conferred by ICMR in 2007.
6. ICMR International fellowship for Senior Biomedical Scientists for the year 2006.
7. Awarded Courtesy Fellowship by CBER, FDA, USA in Sep 2005.
8. Awarded fellowship by National Foundation of Infectious Diseases, USA in 2005.
9. Granted ICMR Award for Excellent Research output in July 2004.
10. Silver Jubilee award by Indian Association of Medical Microbiology in 2003.
11. Awarded Courtesy Fellowship by CBER, FDA, USA in Dec 2003
12. Kshanika Oration Award, a National award for Eminent Woman Scientist, conferred by Indian Council of Medical Research in 2002.
13. National Science Talent Scholarship awarded by N.C.E.R.T. , New Delhi

Patents

1. Awarded US Patent No. 6,855,522, in 2005, for "Species-specific PCR assay for detection of Leishmania donovani in clinical samples of kala-azar and post kala-azar dermal leishmaniasis".
2. US patent no. 20060240046 for "Live attenuated *Leishmania* vaccines"

Membership of professional associations

1. Society for Parasitology, India (Life member).
2. Society of Biological Chemists, India (Life member).
3. Association of Clinical Biochemists of India (Life member)
4. Indian Association of Medical Microbiologists, India (Life member
5. Indian Immunology Society, India (Life member).

Publications

Publications in indexed foreign journals	72
Publications in indexed Indian journals	07
Publications in Proceedings	60
Chapter in Books	07
Total	146