CHEMOTHERAPEUTIC EFFICACY AND SAFETY OF DIMINAZENE ACETURATE–CHLOROQUINE SULPHATE COMBINATION AS AN ANTILEISHMANIAL DRUG IN BALB/c MICE

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Thesis submitted in partial fulfilment of the requirements for the award of the degree of Master of Science (Applied Parasitology) in the School of Pure and Applied Sciences of Kenyatta University

October, 2014
DECLARATION

This thesis is my original work and has not been presented for degree or any other award in any other university.

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DEDICATION

This work is dedicated to my wife Jerris Wambua for her invaluable support during the entire period. Parents Joseph Mwololo and Magdalene for their support, encouragement and financial assistance. They have taught me that I can achieve anything if I put my mind to it. I also dedicate it to my daughters and Siblings who have given me encouragement throughout my study period.
IV

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<thead>
<tr>
<th>Acronym</th>
<th>Term</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AmphotB</td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette Guerin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Chq</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CL</td>
<td>Cutaneous leishmaniasis</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon (IV) oxide</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichloro-diphenyl trichloroethane</td>
</tr>
<tr>
<td>Dim</td>
<td>Diminazene</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FGT</td>
<td>Formal gel test</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Concentration inhibiting parasite growth by 50%</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
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<td>IL 12</td>
<td>Interleukin 12</td>
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<tr>
<td>IPR</td>
<td>Institute of primate research</td>
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<tr>
<td>ITNs</td>
<td>Insecticide treated nets</td>
</tr>
<tr>
<td>LATEX</td>
<td>Latex agglutination test</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Lethal dose</td>
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<tr>
<td>MCL</td>
<td>Mucocutaneous leishmaniasis</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SbV</td>
<td>Pentavalent antimonials</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TH1</td>
<td>T-helper 1</td>
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<tr>
<td>VL</td>
<td>Visceral leishmaniasis</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Leishmaniasis are a complex disease caused by at least 22 different species of protozoan parasites belonging to the genus *Leishmania*. They are significant causes of morbidity and mortality in 88 countries around the world. The clinical manifestations range from self-healing cutaneous and mucocutaneous skin ulcers to fatal visceral form. The use of most antileishmanial drug agents has however been limited because of their toxicity, high cost and declined efficacy due to high resistance and relapse rates. To improve the management of leishmaniasis, alternative chemotherapeutic strategies are required. Combination therapy of antileishmanial drugs is currently considered as one of the rational approaches to lower treatment failure rate and limit drug resistance spreading. The objective of this study was to evaluate the safety and efficacy of a combination between diminazene (Dim) and chloroquine (Chq) as a potentially effective, low cost chemotherapy against visceral leishmaniasis. The experiment involved both *in vitro* and *in vivo* evaluations. *In vitro* drug efficacy involved incubation of *L. donovani* promastigote cultures in 96-well plates with various concentrations of test drugs for 72 hours and calculating drug concentration inhibiting half the number of parasites (IC$_{50}$). Toxicity evaluation involved incubation of Vero cell lines in culture with various concentrations of test compounds for 72 hours and determination of drug concentration killing half the number (LD$_{50}$) of Vero cells. For *in vivo* evaluation, BALB/c mice were infected with *L. donovani* promastigotes and kept for five weeks for establishment of disease. Uninfected (Naive) mice were included in the experiment. Following disease establishment, mice divided in to groups of 8 animals were treated with either, Diminazene (Dim), Chloroquine (Chq), or Diminazene –Chloroquine (Dim-Chq) at doses of 12.5 mg/kg. A fourth group was treated with standard reference drug, Amphotericin B (Amphot B) at doses of 1 mg/kg. While group five mice were not treated and these served as a control. treatments started 7 weeks post infection and were delivered intraperitoneally daily for 21 days. Body weight measurements were taken before infection, 37 days post infection and 7 days after treatment. Seven days after treatment, all mice were sacrificed and IgG antibody responses and splenic parasite loads determined. Antibody responses were quantified by enzyme linked immunosorbent assay (ELISA) while parasite burden was done microscopically by scoring the number of amastigotes in splenic impression smears per 500 cell nuclei. Data were analysed with one way analysis of variance (ANOVA), Tukey-Kramer test, spearman rank correlation test or student t-test. *In vitro* results indicated that dim-chq was at least 9 times more effective than single drugs in inhibiting promastigote growth in culture (IC$_{50}$ = 0.49 ± 0.24). Dim-Chq was as toxic as Dim (LD$_{50}$ = 0.02 ± 0.01). *In vivo* evaluation indicated that, infection of BALB/c mice with *L. donovani* resulted in increase in body weight as measured 37 days post infection (P = 0.007). Of the test drugs, Dim-Chq was associated with the least IgG responses. There was no significant change in body weights for various mice groups following treatments (P = 0.1615). Treatment with Dim-Chq significantly protected mice against visceral leishmaniasis as compared to single drug therapies (P = 0.001). However, Amphot B was more effective against disease than any other drug compound (P = 0.0001). High antibody responses were significantly associated with severe disease (r = 1.000; P = 0.0167). The study concludes that Dim-chq is more safe and efficacious than single drugs and may be developed further for control against human visceral leishmaniasis. In addition, antibody levels may be used as a diagnostic and prognostic tool in this model.
CHAPTER 1: INTRODUCTION

1.1 General introduction
Leishmaniasis is a zoonotic infection that is caused by obligate intracellular protozoa of the genus *Leishmania*. Natural transmission of *Leishmania* parasites is carried out by sand flies of the genus *Lutzomyia* in America or *Phlebotomus*, in the rest of the world (Mandell *et al.*, 2005).

There are three major clinico-pathological categories of leishmaniasis: cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis and visceral leishmaniasis (VL) each caused by distinct species (Enrique *et al.*, 2005). Mucocutaneous leishmaniasis (MCL) is a severe disfiguring disease that usually evolves chronically and is extremely difficult to treat (Marsden, 1986). Cutaneous leishmaniasis (CL) produces a skin ulcer that heals spontaneously in most cases, leaving an unsightly scar (Sohrabi *et al.*, 2005). Visceral leishmaniasis, the most devastating and severe form, is fatal in almost all cases if left untreated. It may cause epidemic outbreaks with high mortality (WHO, 2007).

Leishmaniasis is endemic in 88 countries in the world and 350 million people are considered at risk of infection. An estimated 14 million people are infected, and each year, about two million new cases occur (WHO, 2007). The disease is endemic throughout parts of Africa, India, the Middle East, Southern Europe, and Central and South America (Figure 1.1) and epidemics are also well recognized (Handman, 2001). With the advent of the human immunodeficiency virus (HIV) epidemic, leishmaniasis has surged as a reactivating infection in AIDS patients in many parts of the world (WHO, 1994). Even when coinfected patients receive proper treatment, they relapse repeatedly and the outcome frequently is fatal (WHO, 2007).
Leishmaniasis has been known to be endemic in many parts of Kenya from as far back as early 19th century. Nine sporadic cases of leishmaniasis were reported between 1911 and 1939 (Fendall, 1961). The main endemic foci of visceral leishmaniasis in Kenya are Eastern and the Rift valley provinces (Muigai et al., 1987). Cutaneous leishmaniasis occurs on the eastern slopes of Mount Elgon in Western Kenya, parts of the Rift valley and some parts of Central Kenya among other areas (Mutinga et al., 1975). Some of the counties affected by leishmaniasis include:
Baringo, Isiolo, Meru, Turkana, Laikipia, Kajiado, Machakos, Kitui and Wajir County (Figure 1.2).

Figure 1.2. Geographical distribution of leishmaniases in Kenya (Adapted and modified from Killick-Kendrick, 1999).

Key:  
CL- Cutaneous leishmaniasis,  
VL- Visceral leishmaniasis,  
DCL- Diffuse cutaneous leishmaniasis
Improved control of leishmaniasis will reduce both mortality and morbidity of the disease (WHO, 2007). To date, there is no vaccine against any form of leishmaniasis in routine use anywhere in the world (Mutiso et al., 2012). Vector control measures, such as residual insecticide spraying and the use of insecticide-treated bed nets or curtains, offer effective protection (Reyburn, 2000; Desjeux, 2004). However, treated bed-net programs are poorly implemented in many endemic countries (Thakur, 2000) and are beyond the means of many families in endemic villages (Desjeux, 2004). Current control measures rely on chemotherapy to alleviate disease and on vector control to reduce transmission (Handman, 2001). However, the increasing prevalence of drug-resistant organisms and the tendency for patients to relapse after an initially successful regimen of chemotherapy underscore the need for the development of new effective and safe drugs against leishmaniasis. Combination therapy of antileishmanial drugs is currently considered as one of the most rational approaches to lower treatment failure rate and limit the spreading of drug resistance (Guerin et al., 2002; Gazanion et al., 2011). In a recent drug development study against leishmaniasis, diminazene combined with the antimalarial drug, artesunate, was found to be more effective than the single drug therapies in the treatment of murine visceral leishmaniasis (Mutiso et al., 2011). Research on more drug combinations is important as a rationale for selecting an effective compound that has inherent ability to effectively treat leishmaniasis. The objective of this study was to test the potential use of a combination therapy between diminazene (Dim) and chloroquine (Chq) against visceral leishmaniasis.
1.2 Problem statement
Leishmaniasis is a protozoal disease of man that occurs in most parts of the world according to the World Health Organization (WHO, 2007). The annual incidence of new cases is about 2 million including 1.5 million cases of cutaneous leishmaniasis and 0.5 million cases of the potentially fatal visceral leishmaniasis. Current chemotherapeutic agents remain of limited value because of high toxicity, prohibitive cost, and high relapse rates (WHO, 2007; Mutiso et al., 2011). Furthermore, drug resistance in leishmaniasis has been reported in all current drugs (Croft et al., 2006). However, as other methods of control of leishmaniasis including vector and reservoir control and the fact that there is currently no vaccine for the disease current efforts are aimed at developing safe, effective and cheaper drugs. Combination therapy of antileishmanial drugs is currently considered as one of the most rational approaches to lower treatment failure rate and limit the spreading of drug resistance (Guerin et al., 2002). It was therefore important to evaluate the potential use of a combination therapy between diminazene (Dim) and chloroquine (Chq) as an alternative antileishmanial drug agent.

1.3 Justification
The World Health Organization recommends the production of cheaper and effective drugs and to avert the emergence of resistance, drugs should be given in combination (WHO, 2007). Combination therapy between commercially available drugs that are aimed to reduce cost, toxicity and duration of treatments, represents a promising rational alternative (Gazanion et al., 2011). The possible efficacy of Dim-Chq drug combination and its relatively being cheap will contribute towards the eradication of the disease owing to its being relatively affordable to most people. BALB/c mice have
been used successfully as animal model in many experiments and are therefore appropriate in drug testing. (Mutiso et al., 2011).

1.4 Research questions

i. What is the efficacy level of Dim-Chq against \textit{in vitro} promastigotes as well as against murine \textit{L. donovani} visceral leishmaniasis?

ii. What is the toxicity level of Dim-Chq against Vero cells in culture?

iii. What are the levels of IgG antibody responses in BALB/c mice following \textit{L. donovani} infection and treatment with Dim-Chq and control drugs?

1.5 Hypotheses

i. Dim-Chq is neither safe against vero cells nor efficacious against \textit{in vitro} or \textit{in vivo} \textit{L. donovani} parasites

ii. There is no difference in levels of antileishmanial antibody responses in BALB/c mice following \textit{L. donovani} infection and treatment with Dim-Chq or control drugs.

1.6 Objectives

1.6.1 General Objective

To determine the combined therapy of Dim-Chq as a safe and effective option for treatment of Leishmaniasis in Murine model for potential application in human model.
1.6.2 Specific objectives

i. To determine the efficacy of Dim-Chq against *L. donovani* promastigotes and murine visceral leishmaniasis.

ii. To evaluate the safety of Dim-Chq by measuring toxicity levels in Vero cells in culture.

iii. To determine IgG antibody responses in BALB/c mice following *L. donovani* infection and treatment with Dim-Chq.

1.7 Significance of the study

Since there are no effective vaccines against any of the major parasitic diseases, and chemotherapy is the main weapon in our arsenal, there is an urgent need for better drugs against *Leishmania*. The objective of this study was to assess the effectiveness of Diminazene and Chloroquine used singly or in combination in the treatment of leishmaniasis as these drugs are cheaply available. These two drug compounds can be formulated for both oral and parenteral administration. The drugs will be tested against the most severe devastating form of leishmaniasis caused by *L. donovani* in experimentally infected BALB/c. The results of this study will also give a guideline for testing this drug compound against clinical leishmaniasis in humans.
CHAPTER 2: LITERATURE REVIEW

2.1 Leishmaniases

Leishmaniases is a general term used for vector-borne parasitic diseases which are transmitted by sandflies and caused by obligate intracellular protozoa of the genus _Leishmania_. Human infection is caused by about 21 of 30 species that infect mammals. These include the _L. donovani_ complex with 3 species (_L. donovani, L. infantum_, and _L. chagasi_); the _L. mexicana_ complex with 3 main species (_L. mexicana, L. amazonensis_, and _L. venezuelensis_); _L. tropica; L. major; L. aethiopica_; and the subgenus _Viannia_ with 4 main species (_L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis_, and _L. (V.) peruviana_). The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies (WHO/TDR, 2004b; Vandana, 2009).

2.2 Parasite biology

2.2.1 Promastigotes stage

Promastigotes stage of _Leishmania_ exists in the sandfly. They are flagellated and spindle-like in shape and measure 15-20 μm in length and 1.5-3.5 μm in width. The amastigotes transform to promastigotes in posterior midgut of sandfly within hours of bite while the promastigotes transform to amastigotes inside macrophages. In this form, nucleus is situated at the center and kinetoplast transversely towards the anterior end. Promastigotes also exhibit a single and delicate flagellum 15-28 μm in length. These promastigotes are morphologically similar to those grown in culture (Hommel, 1978; Bari, 2012).
2.2.2 Amastigote stage

The amastigote form exits in the macrophages of reticuloendothelial system of vertebrates such as spleen, liver, bone-marrow and lymph node. They are ovoid and non-flagellated form of *Leishmania* which are 3-8 μm in length. The centrally located round/oval nucleus and adjacent but smaller round/rod shaped kinetoplast are distinguishable. The flagellum is not functional in amastigotes and does not extend beyond the cell body; however, there is 'Flagellar pocket' which serves as a site of endocytosis and exocytosis. The cytoplasm contains mitochondria, neutral red vacuoles and basophilic, and volutin granules containing RNA. The organism is surrounded by a double membrane below which is a row of 130-200 hollow fibrils (Bari, 2012).

2.3 Life cycle of *Leishmania* parasites

Leishmaniases are transmitted as *Leishmania* infected female sand flies take blood from healthy people. Two transmission ways are possible: zoonotic which happens when the parasite is transferred from infected non-human reservoir host to healthy individuals or anthropopotic where humans are infected by female Phlebotomine sand flies. Congenital, parenteral and sexual transmissions have also been suspected (Gilles, 1999, Vidyashankar and Agrawal, 2007).

Once the sand fly has been infected, the amastigote migrates to the alimentary canal of the insect where it attaches to local epidermal cells. The parasites mature and differentiate into motile promastigotes and move to the pharynx/or the proboscis of the sand fly. Upon a subsequent blood meal, the infective promastigotes are injected into blood stream of the victim (Figure 2.1). Promastigotes that reach the puncture wound are phagocytosed by macrophages and other types of mononuclear phagocytic
cells and reside in the parasitophorous vacuole (Hazra, 2001; Paredes et al., 2003). Promastigotes transform into amastigotes, which replicate by simple division and are released back into the blood stream, proceeding to infect other mononuclear phagocytic cells and macrophages within the skin, viscera and blood tissues, thus disseminating the disease locally or systematically based on distinct Leishmania species involved (Alvar et al., 1997). Parasite, host and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. The parasites are equipped to evade the digestive enzymes present in the vacuole and they also have a membrane bound molecule, lipophosphoglycan that ensures intracellular survival of the parasite (sacks and Perkins, 1985; Cunningham, 2002; Melhorn, 2004).

Figure 2.1 Life cycle of Leishmania parasites (Adopted from Handman, 2001).
2.4 Mode of transmission

All species are transmitted by small blood-sucking sandflies, notably *Phlebotomus* species in the Old World (Middle-East and Africa) and *Lutzomyia* species in the New World (Central and South America). Infections are confined to tropical and subtropical areas (Convit et al., 2005). Only the females feed on blood. Amastigotes ingested during feeding transform in the midgut or hindgut of insect vectors into promastigotes which multiply by binary fission. The parasites migrate forward to the foregut and proboscis where some become swept away by saliva into the bite site when the fly feeds and are transmitted via bite to the tissues of vertebrate hosts (Bari and Rahman, 2008). The main reservoir hosts for *Leishmania* are domestic animals (for example dogs, cats and horses), peridomestic animals (for example mice and rats) and wild animals (for example rodents, hyraxes, sloths, bats, oppsumes, kangaroos, wolves and foxes) (Bates, 2007; WHO, 2008).

2.5 Epidemiology of leishmaniasis

2.5.1 Global situation

Leishmaniasis is considered endemic in 88 tropical and subtropical countries in Central America, South America, Southern Europe, Asia (excluding South-East Asia), Middle East, and Africa (East and North Africa), with some cases elsewhere where approximately 350 million people live (82% of which are developing countries) (Patz et al., 2005; WHO, 2007). A total of 350 million people are at risk, 82% of which are from developing countries, there are 2 million new cases of leishmaniases annually while 14 million are directly affected by the disease (Ashford, 2000; WHO, 2007b).
There are an estimated 500,000 new cases of visceral leishmaniasis and more than 50,000 deaths from the disease each year. The majority of cases occur in Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil (Chappuis et al., 2007; Shiraz and syed 2007). Cutaneous leishmaniasis is the most common form whose new occurrence is estimated to be 1500,000 each year (Shepherd et al., 2008; Craig, 2011) Cutaneous leishmaniasis caused by L. tropica is common along the shores of Mediterranean, throughout Middle East, Africa and parts of India while cutaneous leishmaniasis caused by L. braziliensis is mainly confined to C. America and S. America (Tonui, 2006; WHO, 2010). The risk factors for the disease are socioeconomic factors (primitive housing, low hygienic situation and houses near vector breeding sites), population movements (for agricultural developments, military activity etc.) and environmental changes. Emergence of HIV/AIDS has also increased reactivity of asymptomatic or previously healed leishmaniasis cases. Currently, Leishmania - HIV co-infections have been reported from at least 35 countries (Ashford and Bates, 1998; WHO, 2007b).

2.5.2 Distribution and vectors of leishmaniases in Kenya

The occurrence of VL is rare compared to CL, and the two diseases do not tend to overlap geographically. Baringo district is the only foci reported where both VL and CL are known to occur in Kenya (Tonui, 2006). Visceral leishmaniasis is found predominantly in the arid, low-lying areas of Turkana, Baringo, West Pokot, Kitui, Meru, Machakos, Tana River, Mandera and Wajir. Visceral leishmaniasis in Kenya is caused by L. donovani (Janjoon et al., 2004). Between 2000 and 2010, Médecins Sans Frontières diagnosed and treated 4,831 patients with visceral leishmaniasis (VL) in the Pokot region straddling the border between Uganda and Kenya. Males between 5
and 14 years of age were the most affected group (Mueller et al., 2014). At Amudat Hospital, there were 4,428 admissions for VL investigation between 2000 and 2006, including 2,461 primary VL, 56 relapses, 4 PKDL, and 1,907 cases ultimately viewed as non-VL cases. In Kacheliba, 2,301 cases of leishmaniasis were diagnosed between 2006 and 2010, including 2,144 primary VL, 81 relapses, and 75 PKDL (Mueller et al., 2014).

Cutaneous leishmaniasis occurs over a wider range of environmental conditions, from semi-arid lowlands to high plateaus. The aetiological agents for CL include *L. major* which has been reported in Baringo and Kitui; *L. tropica* in Laikipia, Samburu, Isiolo, Nakuru and Nyandarua Districts while *L. aethiopica* has been reported in Mt. Elgon areas (Mutinga et al., 1990).

2.6 Pathogenesis

2.6.1 Leishmaniases disease clinical syndromes

Leishmaniases form a range of complex diseases. Depending on strain (s) of the parasite involved in pathogenesis and the immune response established by the host, it can cause clinical symptoms which can range from mild self-limiting cutaneous lesion to fatal visceral diseases. The diseases occur in three forms namely: Visceral leishmaniasis (VL), mucocutaneous leishmaniasis (MCL) and cutaneous leishmaniasis (CL) (Bari, 2012).

2.6.2 Cutaneous leishmaniasis

Cutaneous leishmaniasis has many local synonyms such as Tropical sore, Oriental sore, Aleppo sore or Baghdad sore. Cutaneous leishmaniasis is marked by the
appearance of itchy sores, lesions (ulcers) and swelling of the lymph nodes on arms, nose, legs or face and lower limbs (Vandana, 2009). Over time, the sores develop a red raised border and a depression in the middle, and on healing, the sores leave a scar. Although this form is self healing, it can create a serious disability and permanent depression (Hazra, 2001). A severe, non-healing and relapsing disease called recidivans can also occur due to *L. tropica* (Sundar *et al.*, 2007). Different *Leishmania* species cause Old World versus New World (American) cutaneous leishmaniasis. In the Old World (the Eastern Hemisphere), the etiologic agents include *Leishmania tropica*, *L. major*, and *L. aethiopica*. The main species in the New World (the Western Hemisphere) are either in the *L. mexicana* species complex (*L. mexicana*, *L. amazonensis*, and *L. venezuelensis*) or the subgenus *Viannia* (*L. [V.] braziliensis*, *L. [V.] guyanensis*, *L. [V.] panamensis*, and *L. [V.] peruviana*). The *Viannia* subgenus is also referred to as the *L. (V.) braziliensis* species complex (Bari and Rahman, 2008; Vandana, 2009; Craig, 2011).

### 2.6.2.1 Localized cutaneous leishmaniasis (LCL)

Localized cutaneous leishmaniasis (LCL) in the old world is caused by *L. major*, *L. tropica*, *L. infantum*, and *L. aethiopica*, primarily by the former two. With minor differences, the clinical lesions produced by all these species are similar (Bari, 2012). Solitary or multiple subcutaneous nodules (smooth, soft, mobile and 0.5-2.0 cm in size) may occur proximal to the skin lesions and usually along the axis between the skin lesions and the regional lymph nodes (Convit *et al.*, 2005). Healing usually takes place in 2-6 months in *L. major* infection and 8-12 months in *L. tropica*. The healing is almost always with a scar that is typically atrophic, hyperpigmented, and irregular. Localized cutaneous leishmaniasis can be differentiated as acute (< 6 months
duration), chronic (> 6 months duration) and recidivans. Acute LCL can be further divided into three varieties: nodular-including papules and plaques, nodulo-ulcerative, and ulcerative. These forms may appear singly or as an overlap picture (Bari and Rahman, 2008).

2.6.2.2 Diffuse (Anergic) cutaneous leishmaniasis

Diffuse cutaneous leishmaniasis (DCL) is a polar form of cutaneous leishmaniasis characterized by disseminated nodules, an abundance of parasites throughout the course of the disease, the absence of parasite-specific cell-mediated immune response, and a poor response to antimonials treatment (Bonfante and Barroeta, 1996; WHO, 2009). Diffuse cutaneous leishmaniasis may be caused by *L. amazonensis*, *L. mexicana* and *L. pifanoi* in the new world and by *L. aethiopica* in the Old World, but the disease caused by *L. amazonensis* in Central and South America is more common (Castes *et al* 1984; Desjeux, 2004). Diffuse cutaneous leishmaniasis related to *L. major* has also been reported. The disease usually begins with an initial primary lesion, which disseminates to involve other areas of the skin. The lesions are often scattered over limbs, buttocks, and face. These progress slowly and become chronic; the improvement following treatment is gradual and relapse is a rule. There is no systemic involvement. The histology shows macrophages loaded with amastigotes (Bari and Rahman, 2008; Craig, 2011; Bari, 2012).

2.6.3 Mucocutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis (Espundia) produces extensive disfiguring destruction of mucosa and cartilage of the mouth, nose, ear and pharynx leading to a severe mutilation of the face. The disease may get worse if secondary bacterial or fungal
infection occurs (Davies et al., 2003; Sundar et al., 2007). Mucocutaneous leishmaniasis occurs due to *L. aethiopica* in the Old World and *L. brasiliensis* complex in the New World. Cases due to *L. donovani*, *L. major* and *L. infantum* have also been reported (Paredes et al., 2003).

### 2.6.4 Visceral leishmaniasis (VL)

Visceral leishmaniasis (Kala-azar) is a systemic disease typically caused by *L. donovani* and *L. infantum* (in Europe, Middle East, India, and Africa) and *L. chagasi* (in Central and South America) (Ardehali et al., 1980; Bari, 2012). In visceral leishmaniasis, the parasites are able to invade the spleen and liver, which causes a prolonged splenomegaly. The abdominal swelling of these organs is the most prominent clinical feature of this form of disease. The disease is characterized by irregular fever, weight loss, enlargement of spleen and liver, anemia, leukopenia, skin pigmentation and weakness associated with parasite invasion of spleen, liver, bone-marrow, lungs, oral mucosa, larynx, oesophagus, stomach, small intestine, skin and sex cells (Gilles, 1999; Craig, 2011). The disease may be asymptomatic and self-resolving but usually becomes chronic and is usually fatal if left untreated (Chan-Bacab and Pena-Rodriguez, 2001). Rare VL cases due to *L. tropica* and *L. mexicana* have also been reported (Chappuis et al., 2007).

Some visceral leishmaniasis patients may develop dermal manifestation (depigmented macule, papules, nodules, or infiltrative plaques) called post kal-azar dermal leishmaniasis (PKDL) during or shortly after treatment of VL caused by *L. donovani*. It may also develop on individuals who do not have previous history of VL or its treatment. Post kal-azar dermal leishmaniasis (PKDL) is common in India, Bangladesh and China, less frequent in Africa (regularly in Sudan and Kenya) and
rare in the New World (Charkraberti et al. 1997; Gilles, 1999). The greatest danger with visceral leishmaniasis is its impact on the immune system. After prolonged infection, the host's immune system deteriorates because these parasites directly attack the reticuloendothelial system. As such, people with visceral leishmaniasis become increasingly susceptible to other infections (Craig, 2011; Mishra et al., 2011).

2.7 Diagnosis of leishmaniasis

2.7.1 Diagnosis of Visceral leishmaniasis

As the clinical presentation of VL lacks specificity, confirmatory tests are required to decide which patients should be treated. Such tests should be highly sensitive (>95%) as VL is a fatal condition, but also highly specific because the current drugs used to treat VL are toxic. Ideally, a test should be able to make the distinction between acute disease and asymptomatic infection, because none of the drugs currently available is safe enough to treat asymptomatic infections. Moreover, such tests should be simple and affordable. (Sundar, 2006; Chappuis et al., 2006).

2.7.2 Non-Leishmanial tests

A reduction in the number of red and white blood cells and platelets (pancytopenia) was found to be highly specific (98%) for VL in suspected clinical patients in Nepal but the sensitivity was low (16%) (Boelaert et al., 2004). Marked polyclonal hypergammaglobulinemia (the production of high titres of non-specific antibody), a common finding in VL, can be detected by a formol gel test (FGT; also called the aldehyde test), which is still used in East Africa and Asia because of its simplicity and low cost. However, as the sensitivity of this test is poor (as low as 34%, some experts have recommended its use be discontinued (Sundar, 2003).
2.7.3 Parasite detection

The visualization of the amastigote form of the parasite by microscopic examination of aspirates from lymph nodes, bone marrow or spleen is the classical confirmatory test for VL. Although the specificity is high, the sensitivity of microscopy varies, being higher for spleen (93–99%) than for bone marrow (53–86%) or lymph node (53–65%) aspirates (Zijlstra et al., 1992; Hassan et al., 2001). However, spleen aspiration can be complicated by life threatening haemorrhages in ~0.1% of individuals and therefore requires considerable technical expertise, as well as facilities for nursing surveillance, blood transfusion and surgery. Moreover, the accuracy of microscopic examination is influenced by the ability of the laboratory technician and the quality of the reagents used. The detection of parasites in the blood or organs by culture or by using molecular techniques such as PCR is more sensitive than microscopic examination but these techniques remain restricted to referral hospitals and research centres, despite efforts to simplify them (Reithinger and Dujardin, 2007).

2.7.4 Antibody-detection tests

Several tests that detect specific anti-leishmanial antibodies have been developed, but all have two major limitations. First, though serum antibody levels decrease after successful treatment (Kumar et al., 2002; Chappuis et al. 2006), they remain detectable up to several years after cure. Therefore, VL relapse cannot be diagnosed by serology. Second, a significant proportion of healthy individuals living in endemic areas with no history of VL are positive for anti-leishmanial antibodies owing to asymptomatic infections. The seroprevalence in healthy populations varies from <10% in low to moderate endemic areas, to >30% in high-transmission foci or in household contacts. Antibody-based tests must therefore always be used in combination with a standardized clinical case. (Sundar et al. 2006).
2.7.5 Visceral leishmaniasis

The gold standard for diagnosing visceral leishmaniasis is parasite identification in tissue smears, with splenic aspirate being more sensitive than bone marrow or lymph node aspirates. However, difficulties in obtaining and examining tissues mean that serological methods are increasingly being used. The direct agglutination test, in which stained parasites are agglutinated by serum antibodies, is popular in Iran and Africa, but variation between batches and the high cost of commercially available antigen are limiting factors. In the Indian subcontinent (Sundar et al., 1998) but less so in Europe and Africa (Zijlstra et al., 2001) a rapid strip test is used to detect antibody to rK39 (a conserved antigen of *L. infantum*) and is both sensitive (67-100%) and specific (93-100%). The use of enzyme linked immunosorbent assay (ELISA) using serum has been evaluated for recombinant gp63, the major *Leishmania* surface glycoprotein, as a diagnostic molecule for leishmaniasis in vervet monkeys (Gicheru et al., 1994).

Weak responses in some patients, persistence of antibodies after cure, and presence of antibodies in some healthy individuals are inherent limitations with antibody based diagnostics. Detection of leishmanial antigen in urine through a latex agglutination test (Katex) seems to be promising for both diagnosis and prognosis (Attar et al., 2001). Techniques based on polymerase chain reaction are potentially highly sensitive and specific (Martin-Sanchez et al., 2001) but they need to be made more suitable for field use in terms of cost and user skills required. In patients co-infected with HIV and visceral leishmaniasis, blood smears and culture might yield good results (Davies et al., 2003).
2.7.6 Cutaneous and mucocutaneous leishmaniasis

Touch smears or culture of exudates or scrapings yield good results in the diagnosis of cutaneous leishmaniasis. From a nodule, slit skin smears are often rewarding. Tissue biopsy can be used for impression smears, culture, or animal inoculation, especially for mucocutaneous leishmaniasis (Davies et al., 2003). Although multiple Leishmania species sometimes coexist, species identification is unlikely to be cost effective in the field unless major treatment decisions for cutaneous leishmaniasis become species specific (Davies et al., 2003).

2.8 Control of leishmaniasis

Improved control reduces both mortality and morbidity. It also reduces the role of humans as a reservoir in anthroponotic cycles and makes it possible to avert progression of the disease to complicated forms (WHO, 2007). Control measures aims at eradicating the parasite reservoir in anthroponotic transmission cycles and using control strategies against the vector and zoonotic reservoir in order to break transmission in zoonotic cyles (Lacerda, 1994). Effective control measures must incorporate proper diagnosis before the correct medication can be instituted. This diagnosis may involve parasitological methods of sample observation from patients or samples may be cultured for identification of isolate.

2.8.1 Reservoir control

Dogs are the main reservoir of L. infantum in zoonotic VL. Despite evidence from experimental studies showing a decreased incidence of VL in both dogs and children following serological screening of dogs and killing of sero-positive animals (Ashford, 1998; Fujiwara et al., 2005), the efficiency and acceptability of this control strategy is increasingly being debated. Treating infected dogs is not an effective control strategy
as relapses are frequent and dogs can regain infectivity weeks after treatment (Alvar et al., 1994). Moreover, the widespread veterinary use of VL drugs might lead to resistance in parasites. A new control approach is the use of deltamethrine-treated collars, which reduced the risk of infection in dogs (by 54%) and children (by 43%) in a study conducted in Iran. Vaccination of dogs would nevertheless be the best strategy if an efficacious vaccine can be developed. (Gavgani et al., 2002; Lemesre et al., 2005).

2.8.2 Vector control

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, where the vector (Phlebotomus argentipes) is restricted to areas in and around the home. Following the large scales antimalarial insecticide (dichloro diphenyl trichloroethane (DDT)) spraying campaigns that were implemented in the 1950s, VL almost completely disappeared from the Indian subcontinent (Kaul et al., 1994). Unfortunately, the disease quickly re-emerged when these spraying campaigns were discontinued. Resistance of P. argentipes to DDT remains limited, but has been reported in Bihar. In Sudan and other endemic countries in East Africa, transmission occurs mainly, but not exclusively, outside villages, during shepherding for example. Indoor residual spraying for disease control is therefore unlikely to be as efficient in this region (Hassan et al., 2004).

2.8.3 Insecticide-impregnated materials

The use of insecticide- treated bed nets (ITNs) could concomitantly prevent VL and other vector-borne diseases, such as malaria and Japanese encephalitis (Boelaert et al 2000). There is limited evidence that bed nets provide protection against VL. Case-control studies conducted in Bangladesh and Nepal showed that sleeping under a non-
impregnated bed net during the warm months was a protective factor against VL (odds ratio = 0.20, \( p = 0.001 \); odds ratio = 0.69, \( p = 0.01 \), respectively) (Bern et al., 2000). 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 (Dye, 1996; Ritmeijer et al., 2007). A large prospective randomized controlled trial testing the efficacy of long-lasting ITNs to prevent \( L. \) Donovani infection and VL is underway in Nepal and India (see the Kalanet Project website). Depending on the sleeping traditions of the population and the biting habits of the local vector, other insecticide-impregnated materials such as curtains and blankets should be evaluated for use in VL prevention, as some have been shown to provide efficient protection against cutaneous leishmaniasis (Reyburn et al., 2000).

2.8.4 Vaccines

The observation that spontaneous or drug-induced recovery from CL or VL is accompanied by solid immunity against reinfection provides a rational basis for vaccine development. This fact led to the traditional practice of using live parasites recovered from skin lesions to induce lesions in preferred body sites to prevent disease on reinfection, a process called leishmanization. Such a practice dates back at least 2000 years (Hemplemann, 2007; Modabber, 1987). Nearly 1.2 million people in Iran between 1982 and 1986 received such a live vaccine (Modabber, 1989). Approximately 50% of those who received this vaccine developed skin lesions and of those, 93% demonstrated a positive leishman-delayed hypersensitivity skin test, a good field marker of population immunity. Furthermore, a significant decrease in disease incidence was observed, falling from 14% in the non-vaccinated group to 2.5% in the vaccinated group.
The rationale for using heterologous organisms of lower pathogenicity as vaccines against a more virulent species is based on the high level of immunological cross reactivity between species at the humoral and cellular levels, though this has not necessarily translated into cross-species protection. However, the risk of localized disease and dissemination in the context of HIV, together with the impracticality of delivering fresh cultures of a live vaccine in the field, has made the practice of leishmanization obsolete (Mohebali et al., 2004; Olobo and Reid, 1990). An alternative strategy using attenuated organisms allows the development of an immune response closest to that of natural infection, with exposure to a much larger range of antigens than achieved by using more refined subunit vaccines. However, despite pursuing such a strategy for human or experimental murine leishmaniasis using naturally avirulent organisms (Heyneman, 1971), irradiated organisms (Lemma and Cole, 1979) or genetically manipulated organisms (Streit et al., 2001), there has been little success. Similarly, killed vaccines have shown limited immunogenicity and efficacy even when combined with adjuvants (Sharifi et al., 1998).

A contrasting approach has been to investigate the use of individual molecules as human vaccines. A gp63 peptide vaccine was tested successfully in animal models (Spitzer et al., 1999). However, the success of these vaccines in humans has generally been poor due to the failure to elicit adequate cellular immunity, an essential feature for the control of intracellular infections. Due to these failures, there is currently no vaccine against any form of leishmaniasis in routine use anywhere in the world.
2.9 Chemotherapy of leishmaniases

Chemotherapy of leishmaniasis is aimed at minimizing morbidity and mortality associated with the disease. It is primarily based on toxic antimony compounds but when this agents lack efficacy, other second-line drugs are used. Several other drugs are also in trial. The most common therapies for leishmaniasis are pentavalent antimonials: meglumine antimoniate (Glucantime), pentamidine (aromatic diamidine) and sodium stibogluconate (SbV) (Pentostam). Others that have been used recently include: liposomal amphotericin B, (AmBisome), paromomycin (Humatin) and miltefosine (Miltex) (Blum et al., 2004). Cutaneous leishmaniasis which forms the bulk of the infection has a self healing nature. Nevertheless, the disease is associated with great human suffering and loss of life where it occurs. Even though lesions may heal eventually in absence of treatment, the process is often long and produces significant scarring, thereby justifying the use of chemotherapy for example topical application of paromomycin (Blum et al., 2004). The goal of treating cutaneous leishmaniasis is to eradicate amastigotes as well as reducing the size of the lesions so that healing will take place with minimal scarring (Dumonteil et al., 2001).

2.9.1 Derivatives of pentavalent antimonial

Pentavalent antimonials (SbV) has become the drug of choice for the treatment of all types of leishmaniasis (Vannier-Santos et al., 2002). The drug can be administered intramuscularly or intravenously, and is distributed in high concentration in the plasma, liver and spleen. To date, the precise mechanism of action of antimonials remains an enigma and their antileishmanial action probably depend on the in vivo reduction of SbV form to a more toxic SbIII form, due to that only amastigotes are susceptible to the SbV (Berman et al., 1989. A general consensus is that SbV acts
upon several targets that include influencing the bioenergetics of Leishmania parasite by inhibiting parasite glucolysis, fatty acid beta-oxidation and inhibition of ADP phosphorylation (Chakraborty et al., 1988; Sen and Majumder, 2008). It has also been reported to cause non specific blocking of SH groups of amastigote proteins and cause inhibition of DNA topoisomerase I (Wyllie et al., 2004). More recently, it was demonstrated that antimony can alter the thiol-redox potential in both forms of parasite by actively promoting efflux of thiols, glutathione and trypanotione, thus rendering the parasite more susceptible to oxidative stress (Ameen, 2007).

The long course treatment allows antileishmanial levels of the drug to accumulate in tissues, particularly in liver and spleen. The treatment with antimonials causes several side effects, such as: nausea, abdominal pain, myalgia, pancreatic inflammation, cardiac arrhythmia and hepatitis, leading to the reduction or cessation of treatment (Thakur et al., 2004). Currently, several limitations have decreased the use of antimonials: the variable efficacy against CL and VL, as well as the emergence of significant resistance has been increased (Croft and Coombs 2003). The recommendations have replaced the antimonials with amphotericin B in refractory zones (Sundar et al., 1998). Second, new generic of Pentostam have been produced with the aim to decrease the high cost of the treatment. However, caution must be exercised before using SbV from new manufacturers as bad batches because of caused fatal cardiotoxicity (Croft et al., 1987). Intralesional administration can be a choice but each lesion has to be injected individually and do not prevent the potential dissemination of infection (Singh, 2004).
2.9.2 Amphotericin B

Amphotericin B is a macrolide polyene antifungal antibiotic agent, discovered from a bacterium: *Streptomyces nodusus*, actinomycetes obtained from the soil of Orinoco River in Venezuela. In early 1960s it was demonstrated to have antileishmanial activity (Thakur *et al.*, 2004). The antileishmanial activity of amphotericin B is attributable to its selectivity for 24 substituted sterols, namely ergosterol vis-a-vis cholesterol, the primary sterol counterpart in mammalian cells eventually helping to increase drug selectivity towards the microorganism. However, higher concentrations triggers cationic and anionic influx via formation of aqueous pores resulting in cell lyses (Berman *et al.*, 1998; Ramos *et al.*, 1996). Serious adverse reactions have been displayed by the treatment with amphotericin B, including fever with rigor and chills, thrombophlebitis and occasional serious toxicities like myocarditis, severe hypokalaemia, renal dysfunction and even death. Its use requires prolonged hospitalization and close monitoring (Balaña-Fouce *et al.*, 1998).

Amphotericin B has excellent leishmanicidal activity and constitutes an option in patients that showed resistance to treatment with antimonials. The major limiting factor about the use of this drug is due to their toxicity (Convit *et al.*, 1987). Currently, toxic effects of amphotericin B have been largely ameliorated with the advent of lipid formulations (Thakur *et al.*, 1996). Among the lipid formulations, AmBisome is the best tested and some studies demonstrated the successful in patients with CL and VL, particularly in areas where antimonials resistance has been detected. AmBisome have been considered as a high effective, non-toxic form of treatment for VL when administered in a short course (Solomon *et al.*, 2007).
2.9.3 Pentamidine

Aromatic diamidines were first synthesized as hypoglycemics drugs and their chemotherapeutic profile against antiprotozoal therapy was early discovered. It was originally used in the treatment of African Trypanosomiasis and since 1939, its activity against Leishmania infections was demonstrated (Balaña-Fouce et al., 1998). Pentamidine acts on the genome of parasite by hampering replication and transcription at the mitochondrial level. Polyamines are substituted at nucleic acid binding sites, which preferentially bind to kinetoplast DNA (Mishra et al., 2007). Commonly, the treatment with pentamidine causes myalgias, pain at the injection site, nausea, headache and less frequently result in a metallic taste, a burning sensation, numbness and hypotension. Reversible hypoglycemia occurs in about 2% of cases. It causes irreversible insulin dependent diabetes mellitus and death. The cure rate associated with low dose of pentamidine, given for a short period, makes it an attractive alternative for CL in antimonies treatment failure cases. In general, the use of this drug has declined due to their low efficacy and toxicity (Sundar and Chatterjee, 2006).

2.9.4 Miltefosine

The entry of miltefosine into the therapeutic armamentarium of leishmaniasis is considered as a landmark event as for the first time, an orally effective antileishmanial agent had been identified. The antileishmanial mechanism of action of this compound can be extrapolated from its effect on mammalian cells, where it causes modulation of cell surface receptors, inositol metabolism, phospholipase activation, protein kinase C and other mitogenic pathways, eventually culminating in apoptosis (Jha et al., 1999; Sundar and Murray, 2005).
Adverse effects of miltefosine include gastrointestinal disturbances and renal toxicity. Fortunately, these symptoms are reversible and they are not a major cause for concern. As miltefosine is teratogenic, it is contraindicated in pregnancy and women of child bearing age group, not observing contraception (Sundar and Chatterjee, 2006). Miltefosine has been hailed as a novel oral drug for treatment of VL, with successful application in immunocompetent and immunocompromised patients and perhaps the most significant recent advances (Sundar et al., 2002).

2.9.5 Paromomycin

Paromomycin 6 (aminosidine) is an aminoglycosidic aminocyclitol produced by Streptomyces riomosus var. Paromomycinus. It is effective against a wide range of bacteria and protozoa (Thakur et al., 1992; Tracy et al., 2001). The drug is poorly absorbed into systemic circulation after oral administration, but rapidly absorbed from intramuscular sites of injection. Peak concentration in plasma occurs in 30-90 min and its apparent volume of distribution is 25 % of body weight. The half-life varies between 2 and 3 hours in patients with normal renal function. Their clearance of the drug is almost entirely by glomerular filtration (Maarouf et al., 1998).

It binds to the 30S ribosomal subunit, interfering with initiation of protein synthesis by fixing the 30S-50S ribosomal complex at the start codon of mRNA, leading to accumulation of abnormal initiation complex (Sundar and Chakravarty, 2008). In parallel, experimental evidences have shown that paromomycin promoted ribosomal subunit association of both, cytoplasmatic and mitochondrial forms, following low Mg2+ concentration, induce dissociation and also cause dysfunction in respiratory systems (Maarouf et al., 1997). The most common side effect associated with the paromomycin is the ototoxicity, as well as problems in liver function (Sundar et al., 2007).
2.9.6 Other Drugs Clinically Used

2.9.6.1. Azoles

The imidazoles and triazoles are well known oral antifungal agents that are well tolerated. They also have antileishmanial activity against certain species as they inhibit 14α-demethylase, a key enzyme in the sterol biosynthesis pathway, thereby interfering with Leishmanial cell membrane biosynthesis. Among them, Fluconazole has been used against *L. major* in Old World and Ketoconazole in the New World against *L. panamensis* and *L. mexicana* (Faris and Maguire, 2002; Alrajhi *et al.*, 2002).

2.9.6.2 Allopurinol

The antileishmanial activity of the purine analogue allopurinol was identified over 30 years ago. Because it had oral bioavailability and it was widely used for other clinical indications, the drug was investigated in clinical trials for CL and VL. However, the results were disappointing. Allopurinol is used as a substrate by various enzymes of the purine salvage pathway of trypanosomatids, and it is selectively incorporated into nucleic acid in the parasite. In recent years, allopurinol was considered as part of a maintenance therapy for canine leishmaniasis (Koutinas *et al.*, 2001).

2.9.6.3 Sitamaquine

Sitamaquine is an orally active 8-aminoquinoline analogue known as WR 6026. Animal studies showed very encouraging results against VL; although in clinical trials it did not show high efficacy after treatment during 28 days (Dietze *et al.*, 2001).

2.9.6.5 Immunomodulators

Cure of leishmaniasis appears to be dependent upon the development of an effective immune response, which activates macrophages to produce toxic nitrogen and oxygen...
metabolites to kill the intracellular amastigotes. This process is suppressed by the infection itself, which down regulates the requisite signaling between macrophage and T cell such as the interleukin (IL) 12, the interferon (IFN) γ and the presentation of major histocompatibility complex (MHC). One alternative in leishmaniasis treatment is the association of antileishmanial drugs with products that stimulate the immune system. The purpose is to enhance the immune response by the activation of macrophages and the increase of the nitric oxide production among other mechanisms to eliminate the infection.

The first report about the use of immunomodulators was the superiority of human IFN-γ as an adjunct antimony therapy for VL, which was demonstrated in Kenya and India (Badaro et al., 1990). Amphotericin B in conjunction of IL-12 or IL-10 was more efficient than monotherapy and led to a reduction of the amphotericin dose (Murray et al., 2003). Other studies have been reported, using immunomodulators like BCG and protein A (Ghose et al., 1999). Nevertheless, the price of immunomodulators is exorbitantly high for poor population (Sundar and Murray, 1995). Recently, a new generation of synthetic immunomodulator drugs has shown potential for Leishmania treatment. A Schiffbase forming compound, Tucaresol, enhances TH1 response and the production of IL-12 and IFN-γ in mice and human in patients with viral infections and cancer. Tucaresol also has activity against infection caused by L. donovani in BALB/c mice and C57BL/6 at a dose of 5 mg/Kg (Smith et al., 2000). Iminoquimod, an imidazoquinoline, is the ingredient of a cream (AldaraTM) used for the treatment of genital warts. This drug has been shown to induce nitric oxide production in macrophages and it was effective in vitro against L. donovani (Buates and Mattashewski, 1999). This field can be more explored with new
products, aiming to validate the use of immunomodulator for treatment of leishmaniasis, particularly in patients infected with strains that can develop ML or other complications.

2.9.6.6 Combined Therapy

After increasing unresponsiveness to most of the monotherapeutic regimens, the combination therapy has found new scope in the treatment of leishmaniasis. The combination of antileishmanial drugs could reduce the potential toxic side effects and prevent drug resistance. Several works have shown that some drugs increase their antileishmanial effect in combination (Bryceson, 2001). Paromomycin has been used extensively in Sudan in combination with sodium stibogluconate for the treatment of VL in a period of 17 days (Melaku et al., 2007). The superiority of this combination has been demonstrated in several studies (Thakur et al., 1995). Combined chemotherapy against VL in Kenya was evaluated using oral allopurinol (21 mg/Kg, three times a day for 30 days) with endovenous pentostam (20mg/Kg once a day). The therapy was efficient, but relapses were found in the first month after treatment (Nyakundi et al., 1994). This clinical evidence demonstrated the superiority of the combination therapy and can be a hope to develop new formulations.

2.9.6.8 Development of new drugs

During the past decades scientists have given new impetus to antileishmanial drug discovery; including (i) knowledge of biology, biochemical pathway and genome of parasite, (ii) a revolution in chemical techniques, (iii) several advances in bioinformatics tools and (iii) a higher number of networks, partnerships and consortia to support the development of new antileishmanial agents. Currently, the developments of both synthetic and natural drugs have relevant importance in the search of new therapeutic alternatives. (Lianet, 2009).
2.9.6.9 Synthetic antileishmanial Compounds

The medicinal chemistry is a recent applied science directed to the development of new drugs that evolved significantly due to recent technological advances, mainly in molecular, structural biology and computational chemistry areas. The generation of structural modifications in an initial molecule (called leading compound) to obtain new derivatives has been one successful approach for the design of new drugs based on known and validated molecular targets in the parasite (Krauth et al., 2003; Linares et al., 2006). The knowledge about the physic-chemical and structural properties of the leading compound and its relation to the pharmacological target or action have provided evidences about the initial pharmacophore group, which is essential to activity (Chagas et al., 2008; Das et al., 2008). Derivatives with pharmacophore group can be obtained with the aim to increase the activity and modulate toxic and pharmacokinetic characteristics of the compound.

This approach together with bioinformatics tools have possibilities the virtual search or in silico of potential drugs. In parallel, the design of specific inhibitors has been explored as a possible means for controlling the parasites growth without damaging the host (Linares et al., 2006). The promising potential targets in Leishmania parasite include topoisomerases, kinetoplast, mitochondria, trypanothione reductase, cisteine protease, fatty acid and sterol pathways (Roberts et al., 2006).

Several synthetic products have demonstrated their antileishmanial potentialities. For example azasterols are inhibitors of 24-methyltransferase, which showed activity against promastigotes of L. donovani and axenic amastigotes of L. amazonensis (Magaraci et al., 2003). New alkyl-lysophospholipid derivatives edelfosine and
ilmofosine, demonstrated high in vitro activity against L. donovani promastigotes and amastigotes (Le pape et al., 2000; Azzouz et al., 2005); nicotinamide is an inhibitor of certain III NAD-dependent deacetylase that caused in vitro inhibition of L. infantum promastigotes and amastigotes; n-acetyll- cysteine, a precursor of glutathione, showed in vivo activity against L. amazonensis in BALB/c mice and 3- substituted quinolines have demonstrated their potential as activators of macrophages and in vitro activity against L. chagasi promastigotes and amastigotes was observed (Tempone et al., 2000).

Screening of several library compounds has been reported with promising results. For example, St. George and colleagues screened a chemical library of 15000 compounds and found three compounds (NSC#: 13512, 83633 and 351520) to be active against amastigotes of L. major and safe to mammalian host (St.George, et al., 2006). The analysis of library is an advance technology since several compounds can be searched to gain information on the chemical class of leaders. The synthetic products have been considered successfully, and some advantages are include cost, time of obtention, novelty and scale-up and low intellectual property complications (Kingstone and Newman, 2005). However, the synthetic molecules can display a high toxicity and only a low of compounds have been evaluated in clinical studies.

2.10 Promising Antileishmanial Natural Products

Many people in rural areas depend largely on popular treatments to alleviate the symptoms, particularly the use of medicinal plants (Chan and Fenner, 2001). The natural products are potential sources of wide chemistry with a remarkable diversity and accessibility in nature. Recently, the Tropical Diseases Program of the World Health Organization (TDR/WHO) with the Drug Discovery Research Program has
considered a priority the pharmacological investigation of plants (Peraza-Sánchez et al., 2007). Extensive studies of activity of natural products against *Leishmania* during the last years have been accumulated. Recently, the most advances in this field have been reviewed (Polonio and Effert, 2008; Tempone et al., 2008), which have listed plants and natural product derived that showed some level of antileishmanial activity. Some studies revealed the potential of new products in microorganism or marine sources, such as a glycoprotein isolated from the sponge *Pachymatisma johnstonii*, which showed a high activity *in vitro* against *L. donovani, L. braziliensis* and *L. Mexicana*. Also aphidicolin a fungal metabolite isolated from *Nigrospora sphaerica*, inhibited the growth of promastigotes and amastigotes of *L. donovani* (Chibale et al., 2000; Kayser et al., 2001). Studies on the evaluation of plants extract from different geographic areas have been reported. Brazilian, Mexican, Colombian and Peruvian (Fournet and Munoz, 2002; Kvist et al., 2006) flora extracts showed the antileishmanial activity of plants used by people from endemic areas of Latin America. The antileishmanial activity of essential oil has been evaluated and details reviewed (Anthony et al., 2003).

The oil of *Croton cajucara*, a plant used in folk Brazilian medicine, causes the inhibition of *L. Amazonensis* and increased the nitric oxide production (Rossa et al., 2003). Nerolidol is a compound present in the essential oils of some plants and inhibits the *in vitro* growth of *L. amazonensis, L. braziliensis* and *L. chagasi*. The mechanism of action of these compounds could be the inhibition of earlier steps of ergosterol synthesis (Arruda et al., 2005).
2.11 Current and future developments

Leishmaniasis is a public health problem in many countries of the World. Currently, chemotherapy is the main weapon to combat the infection. Some drugs are commercially available such as pentavalent antimonial, amphotericin B, pentamidine, miltefosine, aminosidine,azole derivatives, allopurinol, sitamaquine and immunomodulators. New formulations of lipid-associated amphotericin B and ointments with aminosidine have been under evaluation in clinical trials that has given promising therapeutic options together with the combination of recommended drugs (Lianet, 2009).

However, the advances in the pharmacology of leishmaniasis are under constant change due to the need to search for better drugs. The research based on (a) knowledge about genome of the parasite, (b) information of drug used for other infection or pathologies, (c) the synthesis of new compounds using rational design of drugs and (d) compounds isolated from new natural sources; can give a solution more efficient and available for treatment of leishmaniasis. (Lianet, 2009).

2.11.1 First line approach

The first line approach for the treatment of leishmaniasis is administration of pentavalent antimony compounds (Herwaldt, 1999). Historically, thousands of leishmaniasis patients treated with antimonials are successfully cured, but always with the danger of well-documented side effects of heavy metal poisoning (WHO, 1984). In addition, emergence of leishmanial resistant organisms to the pentavalent antimony is well documented and, in some endemic areas of the world, treatment failure has reached a level of 60% - 80% (Grogl et al., 1992). Unfortunately, second line alternative drugs are more toxic than antimonial compounds (Sampaio et al., 1971).
Amphotericin B and Pentamidine have shown reasonably good efficacy results in a series of cases reported, but both have been associated with severe, life threatening organ dysfunction and death (Herwaldt, 1999; Pearson et al., 1999). During the last decade, new formulations of Amphotericin B in a liposome or other lipid-complex drug delivery system have significantly decreased the side effects of Amphotericin based therapy (Thakur et al., 1996). However, the price of the liposome-Amphotericin B preparation is prohibitive for most of the millions of people with leishmaniasis in the tropics (Davidson et al., 1996). The most recent invention, Miltefosine, the only medicine administered orally, is to date licensed only in Colombia, Germany and India. As the possibility of its being teratogenic has not been excluded, it should be used under direct observation. Also, to avert the emergence of resistance, it should be given in combination with other antileishmanial drugs (WHO, 2007). In an attempt to develop new safer and cheap Leishmania drugs, diminazene diaceturate has been tested both in vitro and in the murine model of cutaneous (Macharia et al., 2004) and visceral leishmaniasis (Mutiso et al., 2011). In this study, diminazene was be tested in combination with chloroquine.

2.12 Diminazene and chloroquine

2.12.1 Diminazene

Diminazene (Dim) has trypanocidal and leishmanicidal activities on various strains of trypanosomes (Trypanosoma vivax, T. evansi and T. congolense) and on L. donovani in hamsters (Clement et al., 1992). Berenil has been in use as an anti-trypanosome drug for livestock since 1955. The main biochemical mechanism of Berenil’s trypanocidal actions is thought to be by binding to kinetoplast DNA thereby inducing
complete and irreversible loss of kDNA in certain strains of trypanosomes (Leon and Krassier, 1977; Homeida, et al., 1981). Due to its molecular structure, Berenil has particular affinity for A-T base pairs in circular DNA and kinetoplast DNA. Berenil is not licensed for use in humans because of serious side-effects observed in animals, which include tremors, itching, sweating, convulsions, dyspnea, recumbency and vomiting in camels and decreased blood pressure and diarrhea in dogs (Joubert et al., 2003).

2.12.2 Chloroquine

Chloroquine is a 4-aminoquinoline drug used in the treatment or prevention of malaria. It was discovered in 1934 by Hans Andersag and coworkers at the Bayer laboratories, who named it "Resochin". It was ignored for a decade because it was considered too toxic for human use (Michaelides et al., 2011). During World War II, United States government-sponsored clinical trials for antimalarial drug development showed unequivocally that chloroquine had a significant therapeutic value as an antimalarial drug. It was introduced into clinical practice in 1947 for the prophylactic treatment of malaria (Krafts et al., 2012). Chloroquine for long was the drug of choice for treatment and prophylaxis of malaria due to susceptible strains of P. falciparum, P. ovale, P. vivax and P. malariae in most disease endemic tropical countries.

It has long been used in the treatment or prevention of malaria. After the malaria parasite Plasmodium falciparum started to develop widespread resistance to chloroquine, new potential uses of this cheap and widely available drug have been investigated (Plowe, 2005). As it mildly suppresses the immune system, it is used in some autoimmune disorders, such as rheumatoid arthritis and lupus erythematosus. Chloroquine is in clinical trials as an investigational antiretroviral in humans with
HIV-1/AIDS and as a potential antiviral agent against chikungunya fever. The radiosensitizing and chemosensitizing properties of chloroquine are beginning to be exploited in anticancer strategies in humans (Savarino et al., 2003). Plasmodium and *Leishmania* being protozoans it follows that chloroquine could also be having antileishmanicidal effects.

### 2.12.3 Diminazene-chloroquine combination therapy

Combination therapy of antileishmanial drugs is currently considered as one of the most rational approaches to lower treatment failure rate and limit the spreading of drug resistance (Guerin et al., 2002; Gazanion et al., 2011). In a recent drug development study against leishmaniasis, diminazene combined with the antimalarial drug, artesunate, was found to be more effective than the single drug therapies in the treatment of murine visceral leishmaniasis (Mutiso et al., 2011). However, the synergistic effects of Diminazene-chloroquine combination have so far not been tested despite the two drugs being commonly successfully used against protozoan diseases (Macharia et al., 2004; Krafts et al., 2012). It was imperative that a combination of Diminazene and chloroquine be tested against leishmaniasis in search for safe, low cost and effective drug against this disease. Owing to the pharmacokinetics of the individual compounds the combination may be a promising candidate against *Leishmania*.

### 2.13 Antileishmanial antibody responses

The role of the anti-leishmanial antibody response seen in VL patients is unclear. Patients with active VL have high level of anti-leishmanial IgE, IgM, and IgG (Ghosh et al., 1995; Anam et al., 1999; da Matta et al., 2000; Ryan et al., 2002). Moreover, people living in endemic region are regularly bitten by *Phlebotomus argentipes* and develop anti sand fly salivary antibodies (Barral et al., 2000) indicating that the
sandfly saliva antibody response could be used as a tool for evaluating exposure and/or risk of infection in endemic regions (Clements et al., 2010; Gidwani et al., 2011). It has been suggested that presence of anti-leishmanial antibodies could be predictive of disease (Singh, 2006), but this has not been confirmed. In a longitudinal serological study from an endemic area of Bihar state, India, where 33% of individuals were sero-positive, only 3.5% of them converted to disease. The conversion rate in the sero negative group was not different (2.6%) during the 2 years of follow-up, implying that serological status in healthy individuals cannot predict the disease conversion (Gidwani et al., 2009; Ostyn et al., 2011).

While still limited inability to distinguish between active and past or subclinical infection, antibodies have proven useful in diagnosis of VL disease (Sundar et al., 2002; Sundar and Rai, 2003; Clements et al., 2010; Gidwani et al., 2011). Apart from this, B cells and antibodies have historically not been considered to be of much importance in *Leishmania* infection. High levels of *Leishmania* specific antibodies are observed in patients with VL, whereas CL patients lack *Leishmania* specific antibodies or mount a very weak response. Thus, development of a strong humoral response is more associated with pathology than protection or resolution of disease (Galvao-Castro et al., 1984). In mice, there are accumulating evidences that B cells and antibodies contribute to the VL pathology (Ronet et al., 2008; Deak et al., 2010). Mice lacking B cells were found to be less susceptible to *L. donovani* infection (Smelt et al., 2000) and in cutaneous models of leishmaniasis, immune complex formation and engagement of Fc-receptors have been found to promote parasite replication by driving IL-10 production in macrophages (Kane and Mosser, 2001; Buxbaum and Scott, 2005).
CHAPTER 3: MATERIALS AND METHODS

3.1 Research facility

The *in vitro* evaluations including efficacy and safety tests were carried out in the *Leishmania* Research Program at the Institute of Primate Research (IPR) whereas murine visceral leishmaniasis studies were done at the Immunology and Parasitology Research Laboratory, Department of Zoological Sciences, Kenyatta University.

3.2 Parasites for infection

*Leishmania donovani* strain NLB-065 originated from the spleen of an infected patient from Baringo County, Kenya and is maintained by intracardiac hamster-to-hamster passage at the Institute of Primate Research. A hamster splenic aspirate was cultured in Schneider’s Drosophila Insect medium supplemented with 20% fetal bovine serum and 100 μg/mL of gentamicin at 25°C till stationary phase. Parasites harvested at stationary phase after 6 days of culture were centrifuged at 2500 rpm for 15 min at 4°C and washed three times in sterile phosphate-buffered saline before being counted and used for experimentation.

3.3 Chemicals

Diminazene (Dim) and Chloroquine (Chq) granules were provided by Dr Alain Bourdichon (TropMed, Germany). The compounds were weighed separately, mixed in a 1:1 ratio by weight and used in dosages of 12.5 mg/kg of body weight. Diminazene and Chq were also individually used at dosages of 12.5 mg/kg. Amphoteric B at doses of 1 mg/kg was used as a positive control drug.
3.4 Experimental design

3.4.1 In vitro evaluation against Leishmania donovani promastigotes

Stationary-phase promastigotes harvested as described above were counted and resuspended in a concentration of $2.0 \times 10^6$ parasites/mL in culture medium. With a few modifications, the tests were performed as previously described (Okpekon et al., 2004). The tests were performed in 96-well microtitre plates maintained at 26 °C under 5% CO$_2$ atmosphere. Two hundred microliters of complete Schneider’s Drosophila medium were placed in the wells containing the maximum concentrations of the compounds and 100 μL in the next wells (2 to 12) and controls; 2 μL of compound solutions of 20 mg/mL in distilled water were added to wells number 1 and serial dilutions (ranging from 100 μg/mL to 0.049 μg/mL) in the wells were performed. Hundred microliters of culture medium containing $2.0 \times 10^6$ stationary phase Leishmania parasites/mL were added to each test well. Tests were performed two times each with double replications for each test compound concentration. Parasite observations and counting was done using a haemocytometer. The results were expressed as the concentration inhibiting parasite growth by 50% (IC$_{50}$ ± SD) after 72 h incubation period.

3.4.2 In vivo evaluation against Leishmania donovani

Six to eight week old BALB/c mice of mixed sexes were infected with $1 \times 10^6$ virulent L. donovani strain NLB-065 harvested at stationary phase as previously described. Infected mice were kept for five weeks for symptomatic establishment of VL. Infection was assessed in several splenic impression smears followed by culture. The animals were then divided into five groups of eight mice each and treated with Dim, Chq, Dim-Chq or Amphot B. One group was not treated and it served as a control. The test drugs were given at dosages of 12.5 mg/kg of body weight while the
reference drug. Amphotericin B was given at a concentration of 1 mg/kg of body weight. All doses were administered intraperitoneally for 21 days from week seven post infection. Two weeks following the last day of treatment all mice were sacrificed and parasite numbers determined microscopically by counting the number of amastigotes in Giemsa stained impression smears. Amastigote burden was compared for both treated and untreated mice groups and expressed as number of amastigotes per 500 splenic cell nuclei. The study was carried out following the approval of Kenyatta University Graduate School.

3.6 Toxicological assay

Vero cells were cultured and maintained in minimum essential medium (MEM), supplemented with 10% FBS as described (Ngure et al., 2009). The cells were cultured at 37°C in 5% CO₂, harvested by tripsinization, pooled in a 50 ml vial and 100 μl cell suspension (1 × 10⁵ cell/ml) put in to two wells of rows A-H in a 96-well micro titre plate for one sample. The cells were incubated at 37°C in 5% CO₂ for 24 hours to attach, the medium aspirated off and 50 μL of the highest concentration (10 mg/mL) of each of the test samples serially diluted. The experimental plates were incubated further at 37°C for 48 hrs. The controls used were with medium alone and MTT reagent (10 mL) was added into each well and the cells incubated for 2-4 hrs for establishment of a purple precipitate as observed under a microscope. The medium together with MTT were aspirated off from the wells, after which 100 μL of dimethyl sulfoxide (DMSO) was added and the plates shaken for 5 minutes. Absorbance was measured for each well at 562 nm using a micro-titre plate reader. Cell viability (%) was calculated at each concentration as described by Huq et al., (2004) using the formula:
Cell viability % = (Average absorbance in duplicate drug wells - average blank wells) /Average absorbance in control wells \times 100.

The proportions of cell viability were subtracted from 100% to obtain the percentage cell death.

3.7 Efficacy evaluation

3.7.1 Determination of splenic parasite burden in mice following infection and treatment.

Two weeks following treatment of mice with various drug compounds, all mice groups were sacrificed and parasite numbers determined microscopically from splenic impression smears as described before (Mutiso et al., 2011). Parasite burden were scored as number of amastigotes per 500 splenic nuclei. Drug efficacy was determined by comparing parasite burden data from treated and non-treated infected mice.

3.7.2 Monitoring of body weights

To assess the effect of L. donovani infection in mice as well as drug efficacy in treated experimental animals, body weights were measured at certain time points. Body weights of mice were taken before infection and shortly before treatments were initiated. In addition mice weights were taken midway during treatments and at the end of the experiment. Weights from the treated group were compared with those from the control group to make evaluations on effect of L. donovani infection or the effectiveness of the drug.
3.8 Enzyme-linked immunosorbent assay (ELISA) for antibodies

The assay was performed as described (Mutiso et al., 2009). Briefly, polystyrene Micro-ELISA plates (Nunc, Copenhagen, Denmark) were coated overnight with 100 μL of *Leishmania major* soluble antigen at a concentration of 10 μg/mL, diluted in bicarbonate buffer (pH 9.6). Nonspecific binding sites were blocked with 3 % bovine serum albumin (BSA) in PBS/0.05% Tween 20 buffer (washing buffer) for 1 hr at 37°C. The plate was washed 6 times with washing buffer before the addition of 100 μL of the serum samples and incubation for 2 hr at 37° C. The plate was washed 6 times as above and 100 μl of 1:4000 horse radish peroxidase-conjugated sheep anti-mouse IgG (Amersham) was used as detecting antibody. Tetramethlebenzidine microwell peroxidase substrate was added to the wells and the plate incubated protected from light for 20 minutes before the optical densities were read at 630 nm in a micro-plate reader (Dynatech Laboratories). All sera were tested at a dilution of 1:8, which had been previously determined as the optical dilution for antibody detection by titration.

3.9 Statistical analysis

Data were analysed using GraphPad InStat software utilizing one way analysis of variance (ANOVA) and Tukey-Kramer test statistic as post hoc where applicable. Student t-test was used for paired case analysis while Spearman rank correlation was used for correlation analysis. A *P* value of 0.05 was considered statistically significant. Descriptive statistics were used where applicable. Data were organized into Tables, bargraphs or line graphs.
CHAPTER 4: RESULTS

4.1 *Leishmania donovani* growth curve

Complete Schneider's Insect tissue media containing $1 \times 10^5$ *Leishmania donovani* newly established promastigotes from cultured splenic biopsy tissue from infected BALB/c mice indicated a slow phase of growth or lag phase between day 0 and day 2 of incubation at $26^\circ C$. Promastigotes growth was exponential from day 2 reaching stationary phase on day 6 upon which the cells were harvested for infection, *in vitro* drug testing and antigen preparation. However, some of the cultures propagated further with cell counting once every two days (48 hours) showed declining number of promastigotes from as high as $8 \times 10^5$ promastigotes counted on day 6 to as low as $2 \times 10^5$ promastigotes on day 14 when the incubation was stopped (Figure 4.1).

![Graph of Leishmania donovani growth curve](image)

Figure 4.1: *Leishmania donovani* growth curve for parasites cultivated in Schneider's Insect tissue media.
At the stationary phase, parasites were slender flagellates with nucleus, kinetoplast and flagellum (Figure 4.2).

Figure 4.2: Giemsa stained fixed *Leishmania* promastigotes harvested on day 6 of culture (Magnification X400).

4.2 Weight changes in infected mice

All experimental mice groups showed increase in weights 37 days following infection with virulent *Leishmania* donovani parasites (Table 4.1). Weight increase as a result of growth was calculated to be 0.77 in the naïve group of mice as measured on day 37 following initial weight measurements. Paired t-test analysis indicated significantly higher weights thirty seven days post infection than mice weights measured before infection ($t = 10.446; \text{df} = 5; P = 0.0007$). Following subtraction of 0.77 g from each mice group mean weight taken on day 37 after infection and pairing of this new weight with the initial weights taken before infection, it was interesting to note that, the paired t-test analysis indicated significantly higher ($t = 3.123; \text{df} = 5; P = 0.0262$)
weight in each treatment mice group after infection with virulent \textit{L. donovani} parasites.

Table 4.1: Weights of mice before and after infection

<table>
<thead>
<tr>
<th>Mice groups</th>
<th>Days before and after infection</th>
<th>Mean weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Diminazene (Dim)</td>
<td>27.82</td>
<td>28.84</td>
</tr>
<tr>
<td>Chloroquine (Chq)</td>
<td>26.59</td>
<td>27.98</td>
</tr>
<tr>
<td>Diminazene-Chloroquine (Dim-Chq)</td>
<td>25.52</td>
<td>26.61</td>
</tr>
<tr>
<td>Amphot B</td>
<td>25.86</td>
<td>27.27</td>
</tr>
<tr>
<td>Control</td>
<td>26.11</td>
<td>27.02</td>
</tr>
<tr>
<td>Naive (Not infected)</td>
<td>27.74</td>
<td>28.51</td>
</tr>
</tbody>
</table>

4.3 \textit{In vitro} drug evaluation against \textit{Leishmania donovani} promastigotes

Promastigotes incubated with various drug concentrations showed an upward increase in numbers with decrease in drug concentration. Amongst the test drugs, the combined drug, Dim-Chq was more efficacious as compared to the single drugs (Figure 4.3). Incubation of promastigotes with Dim-Chq (100 µg/ml) reduced their numbers from $2 \times 10^6$ to $1.8 \times 10^5$ and the parasite numbers increased gradually to $41 \times 10^5$ promastigotes when incubated with 0.049 µg/ml of Dim-Chq. The single drugs were less important in the control of promastigotes growth with Dim showing more inhibitory strength than Chq. However, none of the single drugs reduced the number of promastigotes below the initial starting numbers in any of the drug concentrations.
ranging between 100 μg/mL and 0.049 μg/mL. The reference drug, Amphot B remained the most efficacious, killing all the promastigotes at all concentrations from 100 μg/mL to 0.195 μg/mL. The last two concentrations of Amphot B failed to kill promastigotes as there were $28 \times 10^5$ and $32.2 \times 10^5$ parasites in wells incubated with drug concentrations of 0.098 μg/mL and 0.049 μg/mL respectively. Promastigotes in the control wells ranged from $244 \times 10^5$ to $276 \times 10^5$ indicating at least a 12 fold increase in numbers.

![Graph showing in vitro activity of drugs against L. donovani promastigotes. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B (N = 8 mice per group).]
A computation of drug concentration inhibiting parasite growth by 50% \( (IC_{50}) \) indicated that dim-chq \( (IC_{50}) \) was 9 times more effective than Dim \( (IC_{50}) \) alone and 12 times more efficacious than Chq \( (IC_{50}) \) alone in the control of promastigote growth in vitro (Table 4.2). However, the positive control drug, Amphot B, remained more effective \( (IC_{50} = 0.08\pm0.01) \) than Dim-Chq in inhibiting parasite growth as it showed 6 fold strength than the combination drug.

Table 4.2: Inhibition concentration 50 \( (IC_{50}) \) values of various drug compounds against *L. donovani* promastigotes

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC( _{50} ) (mean±SD µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dim</td>
<td>4.254±0.4</td>
</tr>
<tr>
<td>Chq</td>
<td>5.85±0.45</td>
</tr>
<tr>
<td>Dim-Chq</td>
<td>0.49±0.24</td>
</tr>
<tr>
<td>Amphot B</td>
<td>0.08±0.01</td>
</tr>
</tbody>
</table>

4.4 Drug toxicity levels on Vero cells

Drug toxicity, scored as death of vero cells following 72 hour incubation with the various drugs with concentrations ranging from 10 mg/ml to 0.31 mg/ml indicated that both the combination test compound Dim-Chq and Dim alone killed less Vero cells as compared to Chq alone. It was observed that, a concentration of 5 mg/ml of Dim-Chq was more lethal to Vero cells than the higher concentration of 10 mg/ml. Amongst the test drug compounds, Chq alone appeared the most toxic drug with a killing rate of at least 90% at each concentration tested. However, it was notable that
Amphot B was the most toxic compound, killing over 95% of Vero cells at any given concentration (Figure 4.4).

![Figure 4.4: Percentage death of vero cells following incubation with various concentrations of test drugs. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B.]

The lethal dose 50 (LD₅₀) killing half the number of vero cells indicated that both Dim (LD₅₀ = 0.03 mg/ml) and Dim-Chq (LD₅₀ = 0.03 mg/ml) were less toxic requiring to be in a concentration of 0.03 mg/ml to kill 50% of vero cells in a given in vitro test system. Chloroquine (Chq) alone and Amphot B showed the same toxicity strength at 0.02 mg/ml in killing 50% of Vero cells (Table 4.3).
Table 4.3: \( Ld_{50} \) values of various test compounds and Amphotericin B (Amphot B) on Vero cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>( Ld_{50} ) (mean±SD mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dim</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>Chq</td>
<td>0.02±0.05</td>
</tr>
<tr>
<td>Dim-Chq</td>
<td>0.03±0.24</td>
</tr>
<tr>
<td>Amphot B</td>
<td>0.02±0.01</td>
</tr>
</tbody>
</table>

4.5 Antileishmanial antibody responses

All infected mice produced significantly higher antileishmanial IgG antibodies as compared to the naïve (uninfected) mice \((F = 2.848; P = 0.0361)\). Amongst mice groups treated with the test drugs, Dim-Chq induced the least IgG antibodies followed by mice treated with Dim while treatment with Chq alone induced the highest antileishmanial antibodies. It was interesting to note that treatment with Amphot B was associated with lower IgG antibodies as compared to other drug treated mice groups as well as the infected untreated (Control) mice group. The control mice induced higher IgG antibodies than any other infected mice group \((\text{Figure 4.5})\). However, data analysis with One-way analysis of variance (ANOVA) did not indicate any significant difference in IgG OD values for all experimental mice groups \((F = 1.450; P = 0.2528)\).
Experimental animal groups

Figure 4.5: Antileishmanial antibody responses in groups of mice following infection and treatment with various drug compounds. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B (N = 8 mice per group).

4.6 Weight changes in mice following treatment

The mean weights of mice groups taken before treatment and one week post treatment ranged from 26.30 ± 0.45 g being the lowest taken from the Dim-Chq group to 28.18 ± 0.94 g, being the highest taken from the Dim treated group. Weights from other treatment groups were intermediate (Table 4.4). A two tailed t-test for paired case analysis indicated that the pre- and post- treatment weights were comparable (t = 1.715; df = 4; P = 0.1615).
Table 4.4 The weights of various treatment groups

<table>
<thead>
<tr>
<th>Mice groups</th>
<th>Days before and after treatment</th>
<th>Mean weight (g) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diminazene (Dim)</td>
<td>0</td>
<td>28.18 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>28.18 ± 0.94</td>
</tr>
<tr>
<td>Chloroquine (Chq)</td>
<td>0</td>
<td>27.45 ± 0.76</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>27.45 ± 0.76</td>
</tr>
<tr>
<td>Diminazene-Chloroquine (Dim-Chq)</td>
<td>0</td>
<td>26.30 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>26.30 ± 0.45</td>
</tr>
<tr>
<td>Amphot B</td>
<td>0</td>
<td>27.51 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>27.51 ± 0.51</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>28.15 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>28.15 ± 0.52</td>
</tr>
</tbody>
</table>

4.7 Parasitic burden in splenic tissue

Splenic impression smears showed amastigotes both within infected cells and outside cells following bursting of some infected macrophages and release of the intracellular amastigotes outside cells (Figure 4.6a). Both intracellular and extracellular amastigotes were considered for scoring of parasitic burden in splenic tissues.

Figure 4.6a: A Giemsa stained splenic impression smear indicating amastigotes (Magnification X1000).
Two weeks following 21 days daily drug administration into infected mice, amastigotes in splenic tissues from treated mice showed parasitic burden ranging from 1 amastigote per 500 splenic macrophages in the Amphot B treated group to 28 amastigotes/500 cell nuclei in the control mice group (Figure 4.6b). Parasite numbers amongst the test drug treated mice were 9, 20 and 26 amastigotes/500 splenic cell nuclei in the mice groups treated with Dim-Chq, Dim and Chq, respectively. Analysis with ANOVA indicated a highly significant difference in parasitic numbers between any of the treated mice group and the control group ($F = 158.81; P = 0.0001$). Significantly lower parasite numbers were associated with treatment with Dim-Chq than with either Dim or Chq alone ($P = 0.0001$). Between the single drug treatments, Dim treated mice group showed significantly lower parasite numbers than the Chq treated mice ($P = 0.001$). However, treatment with Amphot B was more protective than treatment with any other compound ($P = 0.0001$).
Figure 4.6b: Mean Parasite load (±SE) in groups of BALB/c mice following *L. donovani* infection and treatment with various drug compounds. **Dim**: Diminazene, **Chq**: Chloroquine, **Dim-Chq**: Diminazene+Chloroquine, **Amphot B**: Amphotericin B (n = 8 mice per group).

**4.8 Relationship between antibody responses and parasite burden**

Spearman rank correlation analysis indicated that, there was a positive significant correlation between antibody responses and amastigote numbers in treated and control mice (r = 1.000; P = 0.0167). This relationship indicated that the mice group with strongest IgG antibody response was associated with the highest number of amastigotes while the mice group with the lowest IgG OD value was more protected against visceral leishmaniasis (Figure 4.7).
Figure 4.7: Relationship between antileishmanial responses and paratic burden in mice following infection and treatments. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B (N = 8 mice per group).
CHAPTER 5: DISCUSSION

5.1 Introduction

The World Health Organization has classified the leishmaniasis as a major tropical disease. An effective vaccine against leishmaniasis is not available and chemotherapy is the only effective way to treat all forms of disease. However, current therapy is toxic, expensive and the resistance has emerged as a serious problem, which has compelled the search for new antileishmanial agents. Combination therapy of antileishmanial drugs is currently considered as one of the most rational approaches to lower treatment failure rate and limit drug resistance spreading (Guerin et al., 2002; Gazanion et al., 2011). Furthermore, combination therapy between commercially available drugs that aimed to reduce cost, toxicity and duration of treatments, represents a promising rational alternative (Gazanion et al., 2011). As part of the efforts to develop safe and effective drug against leishmaniasis, this study sought to determine the safety and efficacy of a combination of diminazene and chloroquine in BALB/c mice. The study evaluated the in vitro safety and efficacy of this drug against Leishmania donovani promastigotes as well as the effects of the drug combination of visceral leishmaniasis in the murine system. Results on clinical disease outcome, toxicity, antileishmania antibody (IgG) responses and efficacy are discussed.

5.2 Effect of Leishmania donovani infection on mice body weights

Infections with visceral leishmaniasis caused by L. donovani are associated with weight loss as one of the major symptoms of the disease (Blackwell et al., 2009). In the present study, the significant increase in weight in all infected mice 37 days after L. donovani infection may be attributable to weight increases in the livers and spleens
as a result of parasite multiplication. This may be the case, given that this weight increase was still significant in experimental mice in relation to naïve mice even after subtracting weight changes caused by normal body mass increase. This finding is supported by a recent study which recorded 14 % and 32% weight increase in *L. chagazi* infected B2R/−/ C57BL/6 knock-out (KOB2) mice livers and spleens respectively (Nico *et al.*, 2012). The weight increase in mice used in the present study may be an early event in visceral leishmaniasis infected subjects before substantial damage to the viscera which subsequently results to weight loss.

5.3 *In vitro* drug activity against *Leishmania donovani* promastigotes

There are positive synergistic effects of Dim-Chq combination over the use of its constituent drug compounds in controlling the multiplication of promastigotes in culture. The more than 9 and 12 fold ability of Dim-Chq over Dim and chq alone respectively to inhibit promastigotes growth by half the original numbers was expected. This is because combination therapies are generally more effective than single drug therapies. In a recent *in vitro* study, diminazene combined with artesunate was shown to be at least more than twice as effective compared to the single drug use in inhibiting *L.donovani* promastigotes multiplication (Mutiso *et al.*, 2011). Other studies have reported the advantage of application of combined drug compounds over the use of the single drug chemicals in the development of drugs against leishmaniasis (Guerin *et al.*, 2002; Gazanian *et al.*, 2011). In the present study, the lower value of IC50 for the Dim than Chq drug indicates that the Dim and not the Chq fraction must have contributed more to the potency of the grug combination. However, with the Amphot reference drug indicating a 6 fold strength in killing promastigotes by half
the original number as compared to the combination drug, the *in vitro* parasite killing strength of the Dim-Chq is still too low.

### 5.4 In vitro drug toxicity levels

The extremely high toxicity levels associated with the Amphot B reference drug confirms the fears and limitations of use of current chemotherapy against leishmaniasis and the need of search for safer drugs that can replace the use of current toxic compounds. Indeed, current drugs for leishmaniasis including amphotericin B are highly toxic (Croft *et al.*, 2006; Monzote, 2009; Gazanion *et al.*, 2011; Mutiso *et al.*, 2011). Associating the Dim-Chq compound with less toxicity levels is therefore a desired value but it may be of concern that the Chq fraction in this combination therapy was as toxic as the Amphot B drug.

This Chq toxicity was unexpected based on recent reports that, in the treatment against human cutaneous leishmaniasis, intralesional application of Chq was found to be safe with no association with adverse events (Yasmin *et al.*, 2011). The higher toxicity levels associated with Chq than with Dim in the present report must have contributed to the relatively low LD$_{50}$ value for the combination therapy since Dim was safer when used alone. The safety of diminazene has also been reported in previous murine (Macharia *et al.*, 2004) and canine (Jacobson *et al.*, 1996) *in vivo* studies. However, despite Dim and the combination therapy having the same toxicity levels, the combination drug of Dim and Chq would still be considered superior over the Dim (or Chq) alone based on the extremely low dosage required of the Dim-Chq to kill half the number of disease causing pathogens as compared to the extremely high dosage required to kill half of a given number of health body cells. As a dosage ratio of 1:61 of the drug combination would be required to kill half the number of
promastigotes and half the number of healthy cells, respectively, this combination therapy is still considered safe and can be developed further for control of leishmaniasis.

5.5 Antileishmanial antibody responses

The significant antileishmanial antibody responses observed in all *L. donovani* infected mice may have been an indication of active visceral leishmaniasis. This confirms earlier findings that presence of antileishmanial antibodies could be predictive of disease (Singh and Sivakumar, 2004). Indeed patients with active VL have shown high level of antileishmanial antibodies (Ghosh *et al.*, 1995; Anam *et al*., 1999; da Matta *et al.*, 2000; Ryan *et al*., 2002). With this understanding, and considering the findings that in the present report, mice groups treated with different drugs induced different levels of IgG antibodies, it would be appropriate to suggest that antibody responses may be used for diagnosis and prognosis of visceral leishmaniasis. This is further supported by the high antibody responses associated with the non treated control mice group infected with *L. donovani* parasites. Previous related studies have shown that, in mice, there are accumulating evidences that B cells and antibodies contribute to the VL pathology (Ronet *et al*., 2008; Deak *et al*., 2010) and that mice lacking B cells were found to be less susceptible to *L. donovani* infection (Smelt *et al*., 2000).

5.6 Weight changes in mice following treatment

The lack of a significant difference in the weights of various experimental and control mice groups following treatments may suggest the difficulty in evaluating drug efficacy based on body weight as a clinical parameter in this study. Weight loss is a
symptom of progressive visceral leishmaniasis (Kumar et al., 2002). However, in the present report, the slight increase in mice treated with the Amphot B and the slight reduction in weights in all other mice groups does not seem to give a clear indication of reliability on weight as a parameter to classify disease severity. Furthermore, the duration of this study from infection through treatment to termination of the experiment may not have been long enough for mice to develop severe disease where body weight would be significantly affected by disease severity.

5.7 Parasitic burden in splenic tissue from mice following treatment

The significant protection against visceral leishmaniasis in mice treated with Dim-Chq as opposed to the Dim or Chq single drug therapy was an indication of the advantages of drug combination in the development of drugs against diseases. This observation may be partly due to different modes of action of combined drugs which may effectively reduce parasite resistance. This is in consistent with the general agreement that combination therapy of antileishmanial drugs is one of the most rational approaches to lower treatment failure rate and limit drug resistance spreading (Guerin et al., 2002; Gazanion et al., 2011). As with the results on activity of these test compounds on promastigotes, Dim appeared to have contributed more than Chq to the effectiveness of the Dim-Chq combination in significantly reducing parasite numbers in the mice group treated with the combination therapy. This may be the case, given the many experiments that have reported association of diminazene with high efficacy in the treatment of cutaneous leishmaniasis in murine and human subjects (Damme and Lynen, 1992; Macharia et al., 2004). However, recently, in the treatment of human cutaneous leishmaniasis intralesional use of chloroquine appeared to be highly effective when compared to meglumine antimoniate (Yasmine et al.,
2011). It therefore follows that a combination of Dim and Chq may be very effective for treatment of visceral leishmaniasis depending on the formulation used. Indeed the more effective compound can be used at a higher concentration than the less effective drug to formulate a combination with a desired efficacy. This is because based on the present findings, if parasitic burden alone was to be used to assess the importance of the combination therapy, the report would invalidate the use of this combination therapy against visceral leishmaniasis and recommend the use of Amphot B due to its significant ability to reduce parasite burden in infected mice.

5.8 Relationship between antileishmanial antibody responses and protection

The significant correlation between antibody responses and parasite loads in study subjects was a confirmation that higher antibody responses are associated with severe disease. Previous studies have indicated that there is a correlation of high antibody titres during active disease and a fall in antibody levels following successful cure (Miles et al., 2005). The present findings may be extended to mean that antibody responses may be reliably used as a diagnostic and/or prognostic parameter in visceral leishmaniasis. The lack of positive association between antibody levels and protection from disease confirms earlier findings that, antileishmanial antibodies, which are produced at low levels in cutaneous leishmaniasis and at very high levels in VL, play no role in protection and that a high antibody level is a marker of progressive disease in visceral leishmaniasis (Melby and Ansteady, 2001; Sharma and Singh, 2009).
CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

I. The combination therapy between diminazene and chloroquine strongly inhibited promastigotes growth in culture and was associated with 9 and 12 folds less IC_{50} as compared to diminazene and chloroquine respectively.

II. Diminazene-Chloroquine combination was as toxic as diminazene alone with LD_{50} of 0.03 mg/ml while Chloroquine alone was as toxic as Amphotericin B with LD_{50} of 0.02 mg/ml.

III. All infected animal groups induced significantly higher antileishmanial IgG antibodies as compared to naïve mice. Although the untreated control mice group was associated with the highest IgG antibody responses, antibody responses in all experimental mice groups were not significantly different.

IV. Post treatment mice groups weights were comparable and were not predictive of disease outcome.

V. Diminazene-Chloroquine combination therapy significantly cured mice against visceral leishmaniasis disease as compared to the single drug therapies. However, amphotericin B was more efficacious than Diminazene-Chloroquine against disease.
6.2 Recommendations

i) A combination therapy between Diminazene and Chloroquine should be evaluated further for potential treatment of human leishmaniasis.

ii) The efficacies of a combination of varying amounts by weight of diminazene and chloroquine needs to be investigated inorder to get the optimum working combination ratio.

iii) The safety of diminazene-chloroquine combination should be investigated in an animal model system before use of this drug compound in humans.

iv) Further research is required to validate the use of antibody levels as a diagnostic and/or prognostic tool in murine leishmaniasis model.
REFERENCES


Heynema, D (1971). Immunology of leishmaniasis. *Bulletin World Health*


World Health Organization (2007). Control of Leishmaniasis. Report by the Secretariat; Sixth World Health Assembly. A60/10 Provisional agenda item 12.3.


APPENDICES

Research project title: Determination of the safety and efficacy of Diminazene - Chloroquine as an antileishmanial drug agent in Balb/c mice.

Appendix 1 Preparation of Leishmania cultures

PROCEDURE

1. RETRIEVING CRYOPRESERVED PROMASTIGOTES-

- Retrieve a sample vial of promastigotes cryopreserved in liquid nitrogen.
- Thaw first on water bath.
- Suspend in 10 ml sterile PBS
- Centrifuge at 2000 rpm at 4°C for 15 minutes and repeat the wash for 3 times.
- Suspend the pellet after wash in 2ml of complete media and incubate at 25°C
- Monitor growth daily

2. TO ISOLATE FROM INFECTED MOUSE

- Anaesthetise infected mouse using ketamine
- Swab off around the lesion area with 70% alcohol and allow to dry
- Aspirate the cellular material from the spleen
- Culture the cellular material in complete M199 media
- Monitor growth daily by observing under microscope (40× objective)
- Harvest at stationary phase by washing 3 times in PBS as in previous description.
- Count for delayed type hypersensitivity leishmania antigen and formalin fixed antigen.
RESULT AND RECORD KEEPING

Count the number of promastigotes using the formula: \( \text{Number counted} \times 10^4 \times \text{dilution factor} \times \text{Volume suspended into estimation} \) the number per ml.

Keep the record of cryopreserved sample vials and number of antigen vials preserved at 70°C (in case of soluble leishmania antigen and formalin fixed antigen.)
Appendix 2 Preparation of impression smears;

Procedure

Place a spleen obtained from a mouse on a tile.

Using a labelled glass slide, press on the spleen to obtain an impression

Allow the slide to air dry

Fix it by dipping in methanol for 30 minutes

Stain the slides by dipping them in a chamber containing giemsa0 stain for 15 minutes

Remove and wash off the excess stain

Allow them to dry ready for viewing under a microscope
Appendix 3 Media preparation

Materials: Serological pipettes, Tuberculin syringes, Safety cabinet, pH meter, M199 powdered media, Gentamycin, Sodium bicarbonate, Double distilled water, 1N sodium hydroxide, 1N HCL.

Procedure

**Dissolve** 15.1 g of powdered media in 900 ml double distilled water in a 1 litre beaker

Stir until it dissolves and add 2.2 g sodium bicarbonate

Allow the solutes to dissolve

Adjust the pH to 7.2 with 1N sodium hydroxide or 1N HCL if necessary

Top up to 1 litre

Filter sterilize through 0.22 micrometer filter and store in culture bottles at 4°C

To prepare complete media add 100 ml fetal bovine serum to 900 ml of incomplete media then add 100 μg/ml gentamycin.