Chapter 5
Chemotherapy and Experimental Models of Visceral Leishmaniasis

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Abstract Visceral leishmaniasis (VL) is a neglected tropical parasitic disease in humans caused by protozoan parasite *Leishmania donovani* and transmitted to humans by the bite of an infected female sand fly, a haemoflagellate vector. According to WHO, every year 0.7–1 million leishmaniasis cases are reported globally, and over 20,000–30,000 deaths occur. Current anti-leishmanial drug (pentavalent antimonials, miltefosine, amphotericin B, pentamidine and paromomycin) therapy is fraught with several problems and causes serious adverse effects, which limit their clinical application. The emergence of drug resistance and non-availability of an effective vaccine(s) against leishmaniasis poses a serious challenge to leishmaniasis treatment and control. Environmental and socio-economic status of people like deforestation, global warming and poverty exacerbates both parasite survival and disease progression. Pentavalent antimonial-resistant strains of *L. donovani* are rampant in Bihar, a highly endemic zone of VL in India. Development of co-infections (HIV-VL and Malaria-VL) often leads to poor diagnosis and treatment. There are no proper prognostic and diagnostic markers for VL. Therefore, there is an urgent need for the development of new anti-leishmanial drugs for the treatment and control of devastating VL. Effective immunotherapy/immuno-chemotherapy is considered as a viable alternative to chemotherapy. Cytokines (granulocyte-macrophage colony-stimulating factor, interferon-γ and interleukin-12) both stand-alone and in combination with current anti-leishmanial drugs are being thought to reduce the drug resistance and useful in VL treatment. The development and availability of the reliable models for anti-leishmanial drug screening is very much warranted.

Keywords Co-infections · Cytokines · Drug resistance · Experimental models · Immunotherapy · Leishmaniasis · Vaccine

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5.1 Introduction

Leishmaniasis is a neglected tropical disease caused by obligate intramacrophage protozoan parasites of genus *Leishmania* and is transmitted to humans by the bite of infected female phlebotomine sand flies. It affects mainly poor population of underdeveloped and developing countries. There are three main forms of the disease: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) or kala-azar (KA) and mucocutaneous leishmaniasis (MCL). Leishmaniasis overall (including VL, CL and MCL) carries the ninth highest disease burden of all the infectious diseases worldwide (WHO 2015). VL is the most dangerous form of leishmaniasis which is characterised by irregular fever, weight loss, enlargement of spleen and liver and decreased blood cell counts; if untreated, it causes the death of the patient. The causative agent for VL is *Leishmania donovani* in Indian subcontinent and *Leishmania infantum* in Europe, North Africa and Latin America. According to the World Health Organisation (WHO 2015), every year 0.7–1 million leishmaniasis cases are reported globally, over 20,000–30,000 deaths occur, and 50,000–90,000 new VL cases are reported globally; more than 90% of new VL cases are reported in Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan. Leishmaniasis is believed to be the third most prevalent vector-borne diseases (the first two being malaria and lymphatic filariasis). CL is the most common form of leishmaniasis characterised by skin lesions (ulcers) on exposed parts of the body, leaving irremovable scars on the body. Most of the CL cases are caused by *L. major*. According to WHO, every year 0.6–1 million new CL cases are reported globally. The Americas, the Mediterranean basin, the Middle East and the Central Asia contribute about 95% of CL in the world, and more than two thirds of new CL cases are reported in Afghanistan, Algeria, Colombia, Brazil, Iran and Syrian Arab Republic. MCL is another form of leishmaniasis caused by *L. braziliensis*. MCL is characterised by destruction of mucous membranes in the nose, mouth and throat (larynx and pharynx). Plurinational States of Bolivia, Brazil, Ethiopia and Peru contribute about 90% of MCL cases, globally. Post kala-azar dermal leishmaniasis (PKDL) is a consequence of Kala-azar, characterised by the appearance of macular and nodular scars on face and body parts. PKDL cases are mainly reported in East Africa and India. In India, 5–10% of VL patients have been known to develop PKDL. Patients having PKDL can serve as a potential reservoir of VL (WHO 2015; Chappuis et al. 2007).

5.2 Life Cycle of Leishmania Parasite

In order to develop new drugs and combat the growing resistance in leishmania, one should thoroughly understand the life cycle of the parasites, so that viable drug targets can offer better classes of drugs (Fig. 5.1).

The leishmania parasite has a digenetic life cycle, as the parasite shuttles between mammalian host (as amastigotes) and vectors (as promastigotes). In some places,

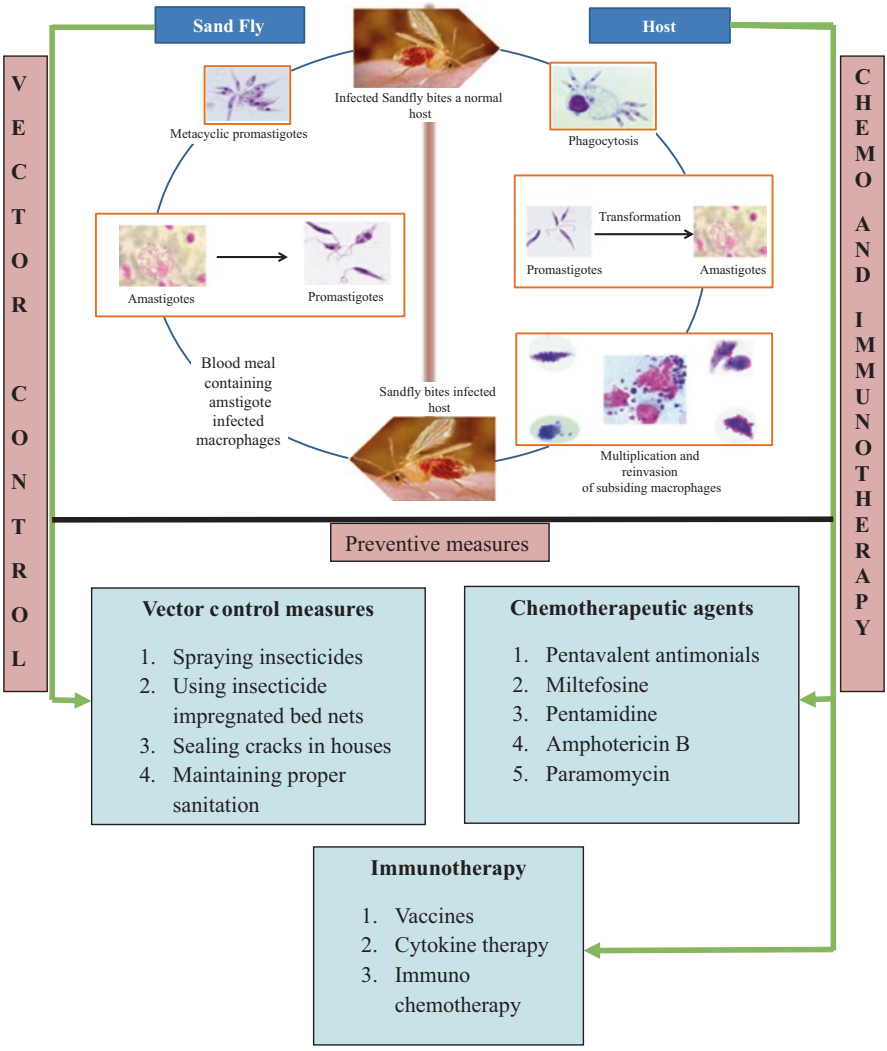

Fig. 5.1 Life cycle of *Leishmania* parasite and potential strategies for control of infection

domestic or wild animals are the reservoirs for the leishmania parasite. Based on this, leishmaniasis can be a zoo-anthroponotic (reservoir-vector-human) and anthroponotic (vector-human) disease. Infected female sand flies inoculate flagellate promastigotes in to the skin of humans; there the promastigotes are phagocytised by neutrophils and macrophages and form phagolysosomes. Within the phagolysosomes of macrophages, promastigotes are transformed into a flagellate amastigote stage which multiply rapidly and reinfect adjacent macrophages of the reticuloendothelial system.

5.3 The Life Cycle of Leishmania Parasite in Vector

The main vector for leishmania is female sand fly belonging to the genus *Phlebotomus* (Old World) and *Lutzomyia* (New World). These are tiny insects having 1.5–2 mm body length and are mainly found in tropical and subtropical regions of the globe. The development of leishmania parasite in vector (sand fly) is mainly confined to the digestive tract only. When the sand flies feed on the blood of a leishmanial-infected mammalian host, the blood meal containing amastigote-infected macrophages is ingested. These amastigotes reach to the midgut of the sand fly. The temperature and pH alterations in the vector's midgut favour morphological transformation of the ingested parasite from amastigote forms to promastigotes forms. The amastigote form transforms into weakly motile pro-cyclic promastigotes (first replicative forms), having short flagellum, and these sluggish pro-cyclic promastigotes are observed in early blood meal and are separated from the midgut by a type 1 peritrophic matrix. After 2–3 days, pro-cyclics slow their replication and differentiate into strongly motile long nectomonad promastigotes, and in later stages these nectomonads move towards the anterior midgut and transform into short nectomonad promastigotes (leptomonads), which enter another proliferative cycle. Promastigotes finally transform into metacyclic stage (infective stage) and reach to the host, when these infective sand flies bite the vertebrate host. Metacyclic promastigotes have small cylindrical body and elongated flagellum (size of flagellum is double to its body size) and having resistance to complement-mediated lysis. Leishmania parasite needs 6–9 days to complete their life cycle in vector (Dostalova and Volf 2012; Kamhawi 2006).

In this chapter, we have mainly focussed on how the factors like environmental and socio-economic status of people, emergence and consolidation of drug resistance to currently anti-leishmanial drugs mainly pentavalent antimonials, development of co-infections of human immunodeficiency virus (HIV-VL and malaria-VL) and diagnostic difficulties in VL exacerbate its prevalence. The strengths and weaknesses of the currently available anti-leishmanial drugs have been described, and the role of immunotherapy with cytokines GM-CSF, IFN-γ and IL-12 in the treatment of VL and several in vitro and in vivo models along with their pros and cons have been discussed.

5.4 Factors Influencing Leishmaniasis Infection

5.4.1 Environmental and Socio-economic Status

Environmental and socio-economic conditions of people are mainly responsible for the spreading of leishmaniasis. Environmental factors like global warming and deforestation favour the growth and survival of vector *Phlebotomus argentipes*. High temperatures in daytime and increased humidity in nighttime are the

favourable conditions for vector growth and disease transmission. A small temperature change has greater effect on parasite growth in the midgut of the sand flies and enhances their infectivity. Due to deforestation there is a decrease in the boundaries between residential areas and forests. So, the people are highly exposed to vectors, and thus relatively more prone to vector-borne disease. American CL has become more prevalent in Central and South America after deforestation. Poor people are mainly affected by leishmaniasis compared to wealthy ones. Poverty, malnutrition, HIV and genetic susceptibility greatly influence the disease prevalence (Kolaczinski et al. 2008). Migration of people from their places to leishmania-endemic areas is one of the major causes of disease spread. Sand flies, mainly *P. orientalises* carrying *L. donovani* parasites, are mainly associated with black cotton soil and *Acacia balanite* forests in North West Ethiopia (Yared et al. 2014). This vector is actively present at nighttime in dried agricultural fields and causes leishmaniasis in agricultural labours, who reside nearer to this area. Poor sanitation, lack of personal hygiene and cracks in houses facilitate vector survival and lead to the spread of leishmaniasis. Spraying insecticides, using insecticide-treated bed nets and sealing of cracks in houses may decrease the chance of the occurrence of leishmaniasis. Goats and bamboo trees highly support the growth and survival of vector *Phlebotomus*; people who reside close to these areas are more prone to risk of infection. Ownerships of goats poses high risk of VL infection, compared to the ownerships of other livestock (Hasker et al. 2012).

5.4.2 *Drug Resistance, a Serious Obstacle for Leishmaniasis Treatment*

The emergence of drug resistance to currently available anti-leishmanial drugs leads to difficulty in the control of leishmaniasis. Low cost and ease of availability of anti-leishmanial drugs in India often leads to misuse of drugs which results in the development of resistance. Improper dosage forms, duration of intake and failure in completing course of treatment are the main reasons for the emergence of *L. donovani* strains resistant to anti-leishmanial drugs in clinical use. In HIV-VL co-infected patients, resistance emerges to anti-leishmanial therapy, and the rate of relapse is high. Pentavalent antimonials are the mainstay of therapy for the treatment of VL, but in India, especially in North Bihar (VL hyperendemic area), resistance has developed to these antimonials. Initially, in the early 1980s, very small doses and less duration (10 mg/kg for 6–10 days) of therapy to VL patients showed resistance. In later years, pentavalent antimonials at the dose of 20 mg/kg × 20 days also showed treatment failure in North Bihar. Thiol molecules cause oxidative stress inside the macrophages and reduce the formation of glutathione, which ultimately inhibits the conversion of pentavalent (inactive form) to trivalent (active form) antimonial, intracellularly. Increased intracellular thiol concentration leads to development of antimonial resistance. The main mechanism for the development of resistance to antimonials is thiol metabolism. The drug efflux transporters also have

main roles in the emergence of drug resistance. Expression of drug efflux transporters like P-glycoprotein and multidrug resistance protein (MDR) results in the development of resistance to antimonials in laboratory strains of leishmania. Miltefosine, used to treat antimony-resistant VL patients, also suffers from the problem of drug resistance. Inactivation of miltefosine-internalising intracellular proteins *L. donovani* miltefosine transporter (LdMT) and *L. donovani* Ros 3 (LdRos3) lead to the development of resistance to miltefosine (Mohapatra 2014). *Leishmania* parasite has also acquired resistance towards lipophilic anti-leishmanial drug amphotericin B. Multidrug resistance (MDR) genes of ATP-binding cassette (ABC) family of promastigotes cause the efflux of amphotericin B which leads to the development of resistance. There are no molecular markers for the identification of drug-resistant strains of *L. donovani*. In vitro intramacrophage amastigote assay is a useful method for the monitoring of drug resistance in leishmaniasis. Therapeutic drug monitoring of VL patients, use of drug combinations with low dose of drugs for shorter time and use of immuno-chemotherapeutics may decrease the chances of the occurrence of anti-leishmanial drug resistance (Maltezou 2009). Identification and elucidation of mechanisms causing resistance is useful for development of newer anti-leishmanial drugs.

5.4.3 Nonavailability of a Proper Vaccine(s)

Presently available anti-leishmanial therapy is costly and causes serious adverse effects. Even though extensive research has been reported in the development of vaccines against leishmaniasis, till now there is no licenced vaccine(s) available around for human use. We are in urgent need for the development of proper vaccine for the prophylaxis and treatment of leishmaniasis. Earlier, leishmanization was a method that has been mainly practised in Soviet Union, Middle East and Israel. In this method, live virulent parasites are inoculated; however, the main drawback is the development of nonhealing cutaneous lesions and immunosuppression in some individuals. Whole-killed (autoclaved) promastigotes and autoclaved parasites along with adjuvant BCG also have been tested as vaccine. It has reduced the occurrence of leishmaniasis, but autoclaved parasites have shown reduced potency with time (Kumar and Engwerda 2014). Live attenuated, dendritic cell-based and DNA-based vaccines that have been tried against leishmaniasis are not fruitful.

5.5 Development of Drugs for Co-infections

Development of co-infections like HIV-VL and malaria-VL leads to the difficulty in the diagnosis and treatment of VL.

5.5.1 HIV-VL Co-infection

VL is a more commonly occurring opportunistic parasitic infection in immunocompromised patients affected by human immunodeficiency virus. It is very common in tropical, subtropical and Mediterranean regions of the globe. The HIV-VL co-infections are highly reported in Indian subcontinent, Western Europe and African regions, where VL is prevalent (Tavora et al. 2015). Both HIV and VL affect T-lymphocytes, macrophages and dendritic cells of the host. The immunosuppression caused by HIV facilitates uncontrolled multiplication of amastigotes in macrophages and accelerates the progression of VL disease and the replication of retrovirus (Lindoso et al. 2014). In vitro co-infection of monocyte-derived macrophages with HIV-1 virus and *L. donovani* promastigotes could hasten parasite growth as compared to the macrophages-infected with *L. donovani* promastigotes alone (Wolday et al. 1998). In HIV-infected patients, both T-cell proliferation and IFN-γ production are impaired, which supports the spread of the parasite in several locations. In HIV-VL co-infected patients, amastigote bodies have been observed in atypical locations including the digestive tract, skin, lungs and tonsils. These atypical symptoms have been more frequently observed in patients having a $CD4^+$ T-cell count of less than 50 cells/mm^3 (Rosenthal et al. 2000). In co-infected patients, HIV-mediated immunosuppression could lead to increased VL relapse rate in immunocompromised patients. Diarrhoea is the main symptom in HIV-VL co-infected patients. The choice of treatment is very difficult for this co-infection because both the disease accelerates the pathology of one another. Highly active antiretroviral treatment (HAART) is the main therapy for HIV-VL co-infected patients that could reduce the VL relapse (Okwor and Uzonna 2013).

5.5.2 Malaria-VL Co-infections

Malaria is a parasitic disease caused by *Plasmodium* species. Kala-azar and malaria are highly endemic in tropical countries like India and Nepal. The immunological status of patients in both the diseases is different. Low blood $CD4^+$ and $CD8^+$ T-cell ratio has been reported in chronic VL-infected patients, whereas in malaria patients this ratio remains unaltered. In co-infected patients, $CD4^+$ T-lymphocyte-mediated protective immunity against malaria was abolished by VL. Malaria cachexia is a condition in which the co-infection of malaria and VL leads to severe weight loss in children with malnutrition. In a case study of malaria-VL co-infected Nepal patient, fine needle aspiration of the left side lymph node showed *L. donovani* bodies in cytological diagnosis (Sah et al. 2002). Migration of VL patients to malaria endemic regions is one of the main reasons for acquisition of co-infections. Diagnosis and treatment of this condition is very difficult. In a case study, Nepalese VL patient was migrated to Terengannu in Malaysia where endemicity of malaria is high and there are patients co-infected with malaria. This co-infection led to difficulty in the

diagnosis and treatment of VL (Ab Rahman and Abdullah 2011). In leishmania-malaria co-infected mice, *Plasmodium yoelii* and *L. mexicana* infections were significantly enhanced compared to mice infected with either parasite alone. Metastatic *L. mexicana* lesions were observed in co-infected mice, compared to *L. mexicana* alone infected mice model (Coleman et al. 1988).

5.6 Diagnosis of VL

The diagnosis of VL is difficult. The clinical features (irregular fever, weight loss, enlargement of the spleen and liver, decreased blood cell counts and anaemia) all resemble the clinical features of other diseases like malaria and tuberculosis, and currently available anti-leishmanial drugs are toxic. The lack of specificity in clinical features of VL needs highly sensitive and specific diagnostic tests (Table 5.1).

5.6.1 Microscopic Detection of Parasite

Microscopic detection of *L. donovani* parasites in the spleen, liver and bone marrow aspirates of VL patient is one of the best methods, but it is a painful procedure, and it requires skilful hand. Detection of *L. donovani* parasite in the spleen is a 95% more sensitive diagnostic method (Sundar and Rai 2002). Culture of bone marrow aspirates is, however, a more sensitive diagnostic technique than microscopy. Aspiration specimens are collected aseptically and cultured in Novy-MacNeal-Nicolle medium or in Schneider's Drosophila Medium supplemented with foetal calf serum. Cultures usually begin to show promastigotes in 2–5 days.

Table 5.1 Diagnostic methods for visceral leishmaniasis

S.No.	Method of detection	Characteristic observations
1.	Microscopic detection method	Identification *L. donovani* amastigotes in the liver, spleen and bone marrow aspirates of VL-infected patients. Parasite detection in spleen is 95% sensitive diagnostic method
2.	Culture method	Bone marrow aspirates of VL patients were cultured aseptically in NNN medium and observed for promastigotes in 2–5 days
3.	Haematological examination	Leucopenia, thrombocytopenia, pancytopenia, haemophagocytosis and anaemia are characteristic haematological features of VL. Estimation of total leucocyte counts, bone marrow cellularity and haemoglobulin content in VL patient is one of the diagnostic method
4.	Indirect fluorescent antibody test (IFAT)	Highly sensitive (96%) and specific (98%). Detects Abs which is present in early stages of infection
5.	rK39 ELISA	Specific diagnosis method for HIV-VL co-infected patients

5.6.2 Haematological Examination

Leucopenia, thrombocytopenia, pancytopenia, haemophagocytosis and anaemia are characteristic haematological features of VL. Estimation of total leucocyte counts, bone marrow cellularity and haemoglobulin content in VL patient is one of the diagnostic methods (Agrawal et al. 2013).

5.6.3 Indirect Fluorescent Antibody Test (IFAT)

IFAT is a specific test which detects antibodies, which are present in the early stages of infection, and these antibodies are not observed after anti-leishmanial drug therapy. It is highly sensitive (96%) and specific (98%), but the requirement of sophisticated laboratory conditions prohibits its application in the field. Other commonly used specific diagnostic tests are ELISA, direct agglutination test, immunochromatographic (ICT) strip test and PCR. rK39 ELISA is one of the best diagnostic method specifically for VL and HIV co-infected patients (Chappuis et al. 2007).

5.7 Biomarkers for VL

5.7.1 Direct Markers

Identification of kinetoplast DNA (kDNA) in clinical samples, and quantitative PCR for the detection of parasite load in VL patient's blood are direct markers of VL. Detection of VL-specific antigen is a predictive biomarker. KAtex is a urine-based latex agglutination assay, which detects a heat-stable low-molecular-weight carbohydrate antigen found in the urine of VL patients (Islam et al. 2004).

5.7.2 Indirect Markers

Indirect marker like macrophage-related marker neopterin, a catabolic product of GTP which belongs to heterocyclic pteridine compound, is synthesised by macrophages after interferon gamma (IFN-γ) activation. Neopterin is a marker for cellular immunity. Neopterin levels are elevated in infectious diseases like HIV, hepatitis B, hepatitis C, tuberculosis and malaria. Serum neopterin levels are elevated in VL patients and return back to normal levels after anti-leishmanial drug therapy (Schriefer et al. 1995; Hamerlinck et al. 2000). Adenosine deaminase (ADA) released from macrophages is mainly involved in purine metabolism. ADA levels are elevated in VL patients. In murine model of leishmaniasis, development of Th1

cytokine (IFN-γ, TNF-α and IL-12) response is associated with control of infection, and Th2 cytokine (IL-4, IL-10 and IL-13) response is associated with disease progression (Kip et al. 2015). IL-18 levels are raised in VL patients and reach to normal levels after chemotherapy (Hailu et al. 2004). Acute-phase proteins like C-reactive protein (CRP) and serum amyloid P component (SAP) are also elevated in active VL patients (Kip et al. 2015).

5.8 Current Chemotherapeutic Agents for VL

5.8.1 Pentavalent Antimonials

Antimony has been used as therapeutics from antiquity. Rai Bahadur Sir Upendranath Brahmachari, an Indian scientist, synthesised urea stibamine (carbostibamide), a pentavalent antimony compound used in the treatment for Indian KA-infected patients in 1920. Professor Brahmachari was nominated for Noble Prize in 1929 for this great discovery, which saved the lives of millions of poor Indian KA-infected patients. In 1936, Schmidt developed a stable, water-soluble pentavalent antimonial, solustibosan. Pentavalent antimonials were first introduced in 1945 as first-line drugs for the treatment of leishmaniasis. Sodium stibogluconate (Pentostam^{Tm}) developed by GSK and meglumine antimoniate (Glucantime) developed by Aventis are the two main antimonial drugs that are used for the treatment of both VL and CL. These drugs are mainly active against intramacrophage amastigote stages of *L. donovani* because these pentavalent antimonials (Sb^{V}) can be reduced to active and toxic trivalent antimonials (Sb^{III}) by amstigotes and not by promastigotes (Haldar et al. 2011). The mode of action of pentavalent antimonials is thought to be the specific inhibition of the DNA topoisomerise-1 of *L. donovani.* Sodium antimony gluconate (SAG)-treated *L. donovani*-infected macrophages releases reactive oxygen species (ROS) and nitric oxide (NO) by activating phosphoionositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK), which are potentially involved in parasite-killing mechanisms (Basu et al. 2006). The treatment schedule is 20 mg/kg/day for 28 days. Long duration of treatment through painful intramuscular injections and tissue accumulation of antimonials causes treatment difficulty. The main serious side effects include vomiting, nausea, anorexia, myalgia, abdominal pain, headache, arthralgia, severe cardiotoxicity, pancreatitis and nephrotoxicity. Reportedly, resistance has developed to these antimonials in Indian subcontinents mainly in North Bihar (Croft et al., 2006b). The combination therapy of sodium stibogluconate (20 mg/kg) and allopurinol (20 mg/kg) for 14–54 days effectively cures the sodium stibogluconate-resistant VL patients; no amastigote bodies were observed in patient's splenic aspirates after 19 days of therapy (Chunge et al. 1985). Anti-leishmanial drugs like SAG, miltefosine and paromomycin have shown immunomodulatory effects like T-cell stimulating ability, enhanced production of IL-12,

TNF-α, NO and ROS on BALB/c mice peritoneal macrophages, directly (Ghosh et al. 2013). Emergence of resistance, poor bioavailability, painful daily injection schedule for long duration and severe side effects are some of the main drawbacks with pentavalent antimonials. Therefore, better improved pentavalent antimonial preparations like safe liposomal-encapsulated antimony-based formulations for targeting intracellular parasite and development of orally active pentavalent antimonials by improving their oral bioavailability with the addition of β-cyclodextrin may be useful in treating leishmaniasis (Frezard et al. 2009).

5.8.2 Miltefosine

Miltefosine, chemically hexadecylphosphocholine, is the first oral drug for the treatment of VL. In the late 1980s, it was developed as an experimental anticancer drug by German scientists. Miltefosine was registered in India in 2002 and has saved many people who were refractory to pentavalent antimonials and effectively treated childhood VL. Miltefosine is active on both the promastigote and amastigote forms of leishmania, in vitro. Miltefosine mainly affects the parasite lipid metabolism by inhibiting the synthesis of phosphotidylcholine and affects cell-signalling pathways and membrane synthesis and thus ultimately causes parasite apoptosis (Sundar and Olliaro 2007). Miltefosine shows immunomodulatory properties like enhancement of IFN-γ receptors, IFN-γ-induced STAT-1 phosphorylation, p38MAP kinase-dependent anti-leishmanial functions and IL-12 dependent Th1 responses in *L. donovani*-infected BALB/c mice (Wadhone et al. 2009). The combined therapy of miltefosine and amphotericin B/paromomycin is highly efficient against antimony-resistant Indian KA patients (Croft et al. 2006a). Miltefosine (25 mg/kg/once or twice weekly, *p. o.*) could suppress the posttreatment recurrence in *L. donovani*-infected T-cell-deficient nude mice and can be useful in the oral maintenance therapy in T-cell-deficient patients mainly observed in HIV-VL co-infected patients (Murray 2000). The combination of antiretroviral drugs (protease inhibitors and reverse transcriptase inhibitors) and miltefosine has tested on *L. infantum*-infected BALB/c mouse peritoneal macrophages and promastigotes. In this, combination of miltefosine and efavirenz (non-nucleoside reverse transcriptase inhibitor) has shown better leishmanicidal activity and may be useful in the treatment of HIV-VL co-infected patients (Costa et al. 2016). The common side effects of miltefosine are vomiting, nausea, diarrhoea and loss of appetite. The main limitation for usage of miltefosine is teratogenicity (causes birth defects in pregnant women). Development of drug combination therapies and immuno-chemotherapy with miltefosine may be a better treatment strategy for decreasing the chance of the emergence of drug resistance.

5.8.3 Pentamidine

Pentamidine isethionate is the most commonly used treatment for *Pneumocystis carinii* pneumonia. After developing resistance to pentavalent antimonials, pentamidine which is a diamidine compound is used as second-line treatment for leishmaniasis. Pentamidine is used as secondary prophylaxis to prevent relapse in four immunocompromised patients (three with HIV-1 and one with idiopathic CD4+ lymphopaenia) with relapsing VL (Patel and Lockwood 2009). The combination therapy of pentamidine (300 mg/kg/day, *i.v*) and fluconazole (200 mg/kg/day, *p.o*) for 3 weeks could effectively reverse the relapse in HIV-VL co-infected patients, previously treated with liposomal amphotericin B and miltefosine (Rybniker et al. 2010). Pentamidine isethionate (two intramuscular injections of 4 mg/kg at 2 days interval) effectively treated *L. braziliensis guyanensis*-infected CL patients in French Guiana (Nacher et al. 2001). Pentamidine mainly acts on the AT bases of kinetoplastid DNA (kDNA) of the parasite. Gastrointestinal discomfort, bronchospasm and increased serum creatinine levels are commonly observed in pentamidine-administered patient. Pentamidine isethionate causes cardiotoxicity, renal toxicity and metabolic disturbances like diabetes mellitus (insulin-dependent diabetes in most cases) (Jha 1983).

5.8.4 Amphotericin B

Development of resistance to conventional pentavalent antimonial therapy in India, mainly in Bihar, where VL is hyperendemic, ensued in the introduction of amphotericin B as a first-line parenteral treatment for VL. In India, amphotericin B is introduced for the treatment of refractory VL due to the failure of existing drugs. Amphotericin B has the ability to sequester the cholesterol in host cell membrane, thereby inhibiting the macrophage-parasite interaction. The mechanism of action is mainly its ability to bind ergosterol in parasite cell membrane (Chattopadhyay and Jafurulla 2011). Amphotericin B effectively treats the *L. donovani*-infected euthymic and nude BALB/c mice and may be useful in the treatment of T-cell-deficient VL patients (Murray et al. 1993). Amphotericin B (0.75–1 mg/kg for 15–20 doses daily or on alternate days) through intravenous infusions showed more protection (nearly 100% cure rate) in VL patients. Painful intravenous route of drug administration is the main drawback for amphotericin B. Single dose of liposomal amphotericin B (5 mg/kg) and followed by 7–14 days of short-course oral miltefosine therapy effectively treated *L. donovani*-infected Indian VL patients, and this combination therapy may be useful in the reduction of development of drug resistance and duration of hospital stay of patients (Sundar et al. 2008). Amphotericin B causes severe adverse effects which include hypokalaemia, nephrotoxicity and myocarditis. These effects require close observation and hospitalisation of patients and raise the cost of therapy. These adverse effects are due to high exposure of free- drug to

systemic circulation and deposition in organs. The lipid formulations of amphotericin B have improved pharmacokinetic properties and targeted delivery to organs like the liver, spleen and bone marrow macrophages, where leishmania parasites reside. Lipid-associated formulations including amphotericin B lipid complex (ABLC), liposomal amphotericin B (L-AmB) and amphotericin B colloidal dispersion (ABCD) were developed for the enhancement of therapeutic efficacy of amphotericin B (Hamill 2013).

5.8.5 Paromomycin

Paromomycin (aminosidine) is an aminoglycoside class of antibiotic with unique anti-leishmanial activity. Paromomycin was first isolated from cultures of *Streptomyces rimosus* having antibacterial activity for the treatment of intestinal infections like amoebiasis and giardiasis (Sundar and Chatterjee 2006). Aminosidine ointment (15% aminosidine and 10% urea in white paraffin) has been reported to cure CL effectively in *L. major*-infected patients in the Islamic Republic of Iran (Asilian et al. 2003). Aminosidine (11 mg/kg/day for 21 days) through intramuscular injections appproved as a first-line treatment for VL in Bihar (Moore and Lockwood 2010). Paromomycin involves with mitochondrial ribosomes and causes respiratory dysfunction in *L. donovani* promastigotes. Paromomycin interacts with 30S ribosomal subunit which leads to the inhibition of RNA and protein synthesis (Maarouf et al. 1997). Paromomycin acts synergistically with pentavalent antimonials. Combined therapy of paromomycin and antimonials has been shown to shorten the time course of therapy and reduces ototoxicity and nephrotoxicity caused by aminoglycoside antibiotics in VL patients in Kenya, India and Sudan (Sundar et al. 2009).

5.8.6 Azoles

Azoles were initially developed as antifungal drugs and after that were reintroduced in anti-leishmanial therapy by hampering the parasite ergosterol synthesis by inhibiting the enzyme lanosterol 14-α demethylase. Azoles actively inhibit leishmania culture growth in vitro. N-substituted azoles such as ketoconazole, fluconazole and itraconazole have more effect on leishmania parasite (Croft et al. 2006a, b). Fluconazole (200 mg capsule daily for 6 weeks) effectively treats CL caused by *L. major* in Saudi Arabian people (Zvulunov et al. 2002). The combined therapy of ketoconazole and allopurinol successfully treated a VL-infected renal transplant recipient who had developed pancreatitis due to the long-term use of sodium stibogluconate (Halim et al. 1993).

5.8.7 Sitamaquine in Clinical Trials: A Hope for Controlling VL

Sitamaquine (WR6026) is chemically (N,N-diethyl-N′-(6-methoxy-4-methyl-quinolin-8-yl)-hexane-1, 6-diamine) an 8-amino quinoline derivative developed by Walter Reed Army Institute of Research (WRAIR) and GlaxoSmithKline (GSK), collaboratively, as a potential compound for the treatment of leishmaniasis. Sitamaquine is an orally active anti-leishmanial agent like miltefosine. Sitamaquine reduces the risk of the development of resistance to leishmania parasite due to short elimination half-life (26 h) in humans (Loiseau et al. 2011). Sitamaquine could reverse miltefosine resistance by modulating LMDR1 (P-gp transporter in Leishmania ABC family involved experimental miltefosine resistance) in multidrug-resistant *L. tropica* line that overexpresses LMDR1 (Perez-Victoria et al. 2011). Sitamaquine binds to anionic polar head groups of phospholipids and accumulates in cytosol of *L. donovani* promastigotes in a sterol-independent manner. Lipid trafficking mechanism of leishmania could not be affected by sitamaquine and has been evidenced by ^{1}H NMR analysis of motile lipid (Loiseau et al. 2011). Sitamaquine induces oxidative stress and increases intracellular calcium levels by inhibiting succinate dehydrogenase in *L. donovani* promastigotes and ultimately causes apoptotic death of the parasite (Carvalho et al. 2011). In phase 2 clinical trial, *L. chagasi*-infected Brazilian patients are cured by the administration of sitamaquine, orally, at the dose of 2 mg/kg/day for 28 days (cure rate 67%; Dietze et al. 2001). In phase 2 clinical trials of sitamaquine in *L. donovani*-infected Kenyan patients showed different cure rates at different doses (1.75, 2.0, 2.5 and 3.0 mg/kg/day for 28 days show cure rates of 92%, 80%, 82% and 91%, respectively), and abdominal pain and headache are the common adverse effect; one patient showed severe renal failure at the doses of 2.5 and 3.0 mg/kg/day (Wasunna et al. 2005). Phase 2 clinical trials of sitamaquine in *L. donovani*-infected Indian VL patients showed 100% cure rate at 2 mg/kg/day dose for 28 days, and vomiting, dyspepsia and nephrotic syndrome are commonly observed adverse effects in Indian patients (Jha et al. 2005) (Fig. 5.2 and Table 5.2).

5.8.8 Cytokine Therapy for Leishmaniasis

The emergence of *Leishmania* parasites resistant to anti-leishmanial drugs and the non-availability of effective vaccine(s) against leishmania pose a serious challenge to leishmania control efforts. Effective immunotherapy/immuno-chemotherapy is considered as a viable alternative to the control of leishmaniasis. Cytokines (GM-CSF, IFN-γ and IL-12) stand-alone or in combination with current anti-leishmanial drugs may reduce the emergence of drug resistance.

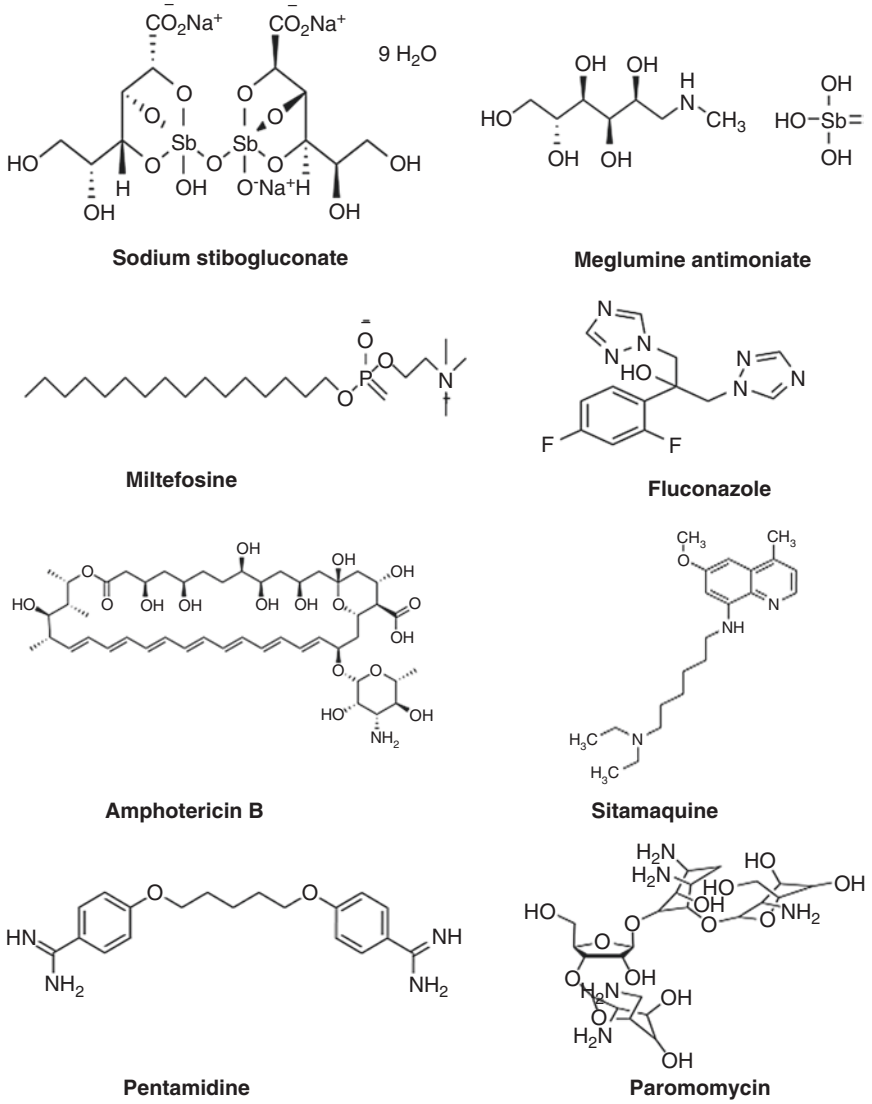

Fig. 5.2 Chemical structures of currently available anti-leishmanial drugs

GM-CSF GM-CSF is an immunoregulatory glycoprotein cytokine having a molecular weight of 23 kDa. In vivo, GM-CSF stimulates haematopoiesis, and in vitro, it stimulates differentiation and proliferation of committed progenitor cells. GM-CSF enhances the phagocytosis of macrophages and improves the host defence (Kaur et al. 2004). GM-CSF was first isolated and purified from a mouse lung-conditioned medium, which stimulated mouse bone marrow cells to proliferate into granulocytes or macrophages or both, by performing in vitro colony-stimulating factor activity

Table 5.2 Currently available anti-leishmanial drugs

S.No	Drugs	Mode of action	Dose	Advantages	Limitations
1.	Sodium stibogluconate	Acts as a prodrug. Converted Sb v to more toxic Sb III form, creates oxidative stress to parasite (macrophage stage)	20 mg/kg/day (i.m.) for 20–30 days	Low cost and easily available	Pancreatitis, acquired resistance in the Indian subcontinent
2.	Miltefosine	Parasite cell death by apoptosis (parasite cell membrane, inositol, PL activation)	50 mg/kg/day (p.o.)	First orally active, currently first-line drug in Indian subcontinent	Hepatic and renal toxicity
3.	Amphotericin B	Binds ergosterol in parasite membranes and creates pores which alter ion balance, increase membrane permeability and cell death	0.75–1.0 mg/kg for 15–20 infusions either daily or alternative days (i.v.)	Effective in antimony-resistant areas	High-cost, prolonged hospitalisation, fever with renal complications and hypokalaemia
4.	Liposomal amphotericin B (AmBisome)	Targeted delivery of drug to infected macrophages	3 mg/kg/day for 5 days (i.v.)	Highly effective, low toxicity, resistance is not documented	High cost
5.	Pentamidine	Accumulates in parasite mitochondria and inhibits kDNA	4 mg/kg/day for 3 times weekly for 15–20 dose (i.m. or i.v.)	Useful in combination of other drugs	Pancreatitis and irreversible IDDM
6.	Paromomycin/ Aminocidin	Protein synthesis inhibitor	11 mg/kg/day for 21 days (i.m.)	Low cost, effective in combination with antimonials	Reversible ototoxicity and lack of efficacy in East Africa

assay (Burgess et al. 1977). Recombinant GM-CSF has been used in the treatment of infectious diseases like malaria and tuberculosis. Immunoadjuvant therapy with rh GM-CSF has shown protective effects in pulmonary tuberculosis patients in phase 3 clinical trials. GM-CSF knockout mice are more susceptible to malaria than wild-type mice infected with *Plasmodium chabaudi* AS, as confirmed by higher peak parasitaemia, recurrent recrudescent parasitaemia and high mortality rate. Combination of rm. GM-CSF and enkephalin fragment peptide Tyr-Gly-Gly has shown protective effects in *Plasmodium berghei*-infected Swiss mice (Kaur et al. 2004). GM-CSF treatment enhances blood monocyte mobilisation, macrophage activation and ameliorates granulocytopenia in *L. donovani*-infected BALB/c mice. Anti-GM-CSF antibody treatment exacerbates the visceral infection, and liver parasite burden was more intensified in *L. donovani*-infected BALB/c mice. During leishmania infection in BALB/c mice, the splenic GM-CSF levels were greatly increased. A 12 kDa *L. donovani* amastigote antigen induced the elaboration of colony-stimulating factors (CSFs) by mouse macrophages, in vitro (Singal and Singh 2005). In leishmaniasis, increased number of GM-CSF cells mediates protection and causes resistance as seen in the case of leishmania-resistant mouse strain (C57BL/6) (Saha et al. 1999). rh GM-CSF has been reported to enhance the intracellular killing of *L. donovani* in human monocyte-derived macrophages in an LPS-independent manner, and the time required for activation of macrophages to show anti-leishmanial effect is very less (rh GM-CSF shows maximal activation at 36 h) as compared to rh IFN-γ, which requires 48–72 h to activate macrophages (Weiser et al. 1987). Purified GM-CSF, isolated from mouse lung-conditioned medium, enhances intracellular killing of *L. tropica* in mouse peritoneal macrophages (Handman and Burgess 1979). The intracellular amastigote killing by rh GM-CSF and M-CSF was more intensified by combining with rh IFN-γ. Combination of rh GM-CSF and sodium stibogluconate effectively treated the CL patients; the lesions were healed in very less time with an antimony dose of 20 mg/kg/day for 20 days. The use of rh GM-CSF in combination with sodium stibogluconate can reduce the dose and duration of antimony therapy and is useful in decreasing the drug toxicity. Combination of rh GM-CSF (10 mμg/ml in 0.9% saline is reapplied topically, and dressings were changed thrice/week for 3 weeks) and antimonials (20 mg/kg/day for 20 days) successfully treated the refractory CL patients (Almeida et al. 1999). A combination of GM-CSF (5 μg/kg/day for 10 days) and pentavalent antimony (10–20 mg/kg/day for 20 days) treatment can rapidly normalise the neutrophil, eosinophil and monocyte counts and reduced the viral and bacterial secondary infections in patients with acute VL (Badaro et al. 1994). Immuno-chemotherapy with GM-CSF and liposomal amphotericin B effectively treats the VL in VL-HIV co-infected patients (Mastroianni 2004). Transgenic recombinant L. major GM-CSF-expressing promastigotes infected BALB/c mouse peritoneal macrophages release GM-CSF can activate macrophages to release high levels of pro-inflammatory cytokines (IL-1β, IL-6 and IL-18) and chemokines (RANTES/CCL5, MIP-2/CXCL-2 and MCP-1/CCL2) involved in enhanced intramacrophage parasite killing in vitro and in vivo. These transgenic promastigotes delay the lesion development in BALB/c mice (Dumas et al. 2003).

IFN-γ IFN-γ is a pleiotropic glycoprotein cytokine having a molecular weight of 20–25 kDa, enhances host defence and is mainly used in the treatment of infectious diseases like toxoplasmosis, leishmaniasis and tuberculosis (Gallin et al. 1995). T-lymphocytes (Th1 cells, T_C cells and NK cells) are the source for IFN-γ production and activate macrophages for boosting defence against infectious diseases. IFN-γ enhances the intramacrophage leishmanicidal effect by activating macrophages to release Th1-type cytokines. Th1 cytokines are mainly involved in protection, and Th2 cytokines are involved in disease pathology (Sundar and Chatterjee 2006). Leishmania parasite control mainly involves increased levels of IFN-γ by $CD4^+$ T cells and exacerbates the disease condition with the absence of IFN-γ in *L. major*-infected C57BL/6 (resistant) and BALB/c (susceptible) mouse strains, respectively (Kima and Soong 2013). Treatment with monoclonal anti IFN-γ antibodies causes CL in C3H/HeN mice infected with *L. major*, which are naturally resistant to CL. Production of IFN-γ during initial period of host-parasite interactions in *L. major*-infected C3H/HeN mice is the major component of genetic control of natural resistance (Belosevic et al. 1989). Treatment with native human IFN-β and IFN-γ of *L. tropica* major-infected human mononuclear phagocytes has shown anti-leishmanial effects three times more than IFN-β, and IFN-γ released enhanced H_2O_2 levels in culture supernatants in a dose-dependent manner; the H_2O_2 release from monocytes was abrogated by the administration of monoclonal IFN-γ antibody. Leishmania-specific CD4 T cells produce IFN-γ which limits parasite replication in VL patients (Singh et al. 2014). In a pilot study, nine VL patients were treated with IFN-γ for 20 days and cleared 100% parasites in bone marrow aspirates of four patients, and five patients have shown reduced parasites in their bone marrow aspirates (Sundar et al. 1995). Combined therapy with rh IFN-γ and pentavalent antimony successfully treated the formerly untreated VL patients and refractory patients (Sundar and Chatterjee 2006). Immuno-chemotherapy with IFN-γ and pentavalent antimonials effectively treated the previously untreated VL patients and was helpful in reducing the duration of conventional therapy. The common side effects with IFN-γ are fever, body aches and flu-like symptoms. IFN-γ-induced fever can be reversed by the administration of antipyretics.

Interleukin-12 (IL-12) IL-12 is a 70 kDa heterodimeric immunoregulatory cytokine which is made up of two subunits of IL-12p35 (35 kDa) and IL-12p40 (40 kDa), linked by a covalent bond. IL-12 is mainly produced by antigen-presenting cells such as macrophages, dendritic cells, monocytes and neutrophils. IL-12 is helpful in the production of IFN-γ and Th1 type cytokines by inducing differentiation of naive $CD4^+$ T cells to Th1 cells and stimulating natural killer cells. IL-12 can be useful in the immunotherapy of diseases where Th1 response is desirable. Due to the induction of IFN-γ and Th1-type cytokines in phagocytes by IL-12, it can be useful in controlling infectious diseases like bacterial, viral and parasitic diseases (Hamza et al. 2010). IL-12 restores IFN-γ production and cytotoxic response in American VL *(L. chagasi*) characterised by the absence of lymphocyte proliferative response and IFN-γ production. Treatment with exogenous rh IL-12 of

L. donovani lysate-stimulated PBMC from active VL patients has been shown to enhance the production of IFN-γ and anti-leishmanial Th1-type response (Bacellar et al. 1996). IL-12 has been effectively shown to treat the established systemic intracellular infection in *L. donovani*-infected BALB/c mice (Murray and Hariprashad 1995). Recombinant murine IL-12 has been shown to increase protection against *L. major* in infected BALB/c mice (Heinzel et al. 1993). IL-12 regulates the leishmanicidal effects of pentavalent antimonials in experimental VL. Combination of IL-12 and pentavalent antimonials effectively treated the *L. donovani*-infected IL-12p35 knockout mice (Murray et al. 2000). Combination of IL-12 DNA and leishmanial recombinant open reading frame F (rORFF) protein induced protective immunity against experimental VL (Tewary et al. 2006). BALB/c mouse peritoneal macrophages pretreated with rm. IFN-γ (1 ng/ml) and rm. IL-12 (1 ng/ml) have shown resistance to *L. major* promastigotes at early phase of infection (Ota et al. 2008).

5.9 In Vitro and In Vivo Anti-leishmanial Screening Methods

The reliable in vitro and in vivo screening methods should have the good correlation with the clinical condition of the disease.

5.9.1 In Vitro Anti-leishmanial Drug Screening Models

Anti-leishmanial drug discovery requires potential drug screening models for testing leishmanicidal activities of newer drugs. In vitro anti-leishmanial drug screening models are advantageous in testing huge number of compounds within a short period of time. Drug testing is possible on parasitic stages like extracellular promastigote stage (survives and multiplies in sand fly's midgut) and intracellular amastigote stage (survives and multiplies in host macrophages). Requirement of less amount of the test compound, less number of animal usage and consistent and quick generation of results for high number of compounds are some of the main advantages of in vitro models.

Promastigotes as Drug Screening Model Promastigotes can grow in cell-free medium. Promastigotes are generally cultured in simple media like M199, RPMI-1640 and Leibovitz-15 and maintained at 22–26 °C in a BOD incubator. Testing of newer potential anti-leishmanial compounds on promastigotes is simple and a highly popular method. In new anti-leishmanial drug screening method, counted number of promastigotes, generally $1.0–2.0 \times 10^6$ cells/ml in culture medium in the presence and absence of appropriate concentrations of test compounds and kept in a BOD incubator maintained at 26 °C. After 3 days of incubation, promastigote growth inhibition by test compounds is determined and compared with controls,

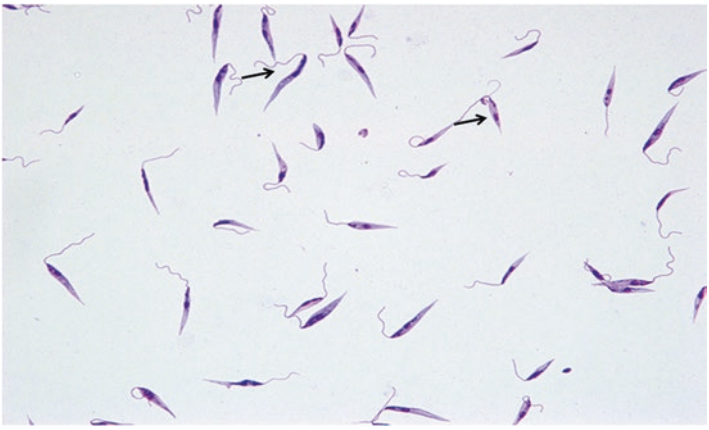

Fig. 5.3 Photomicrograph of *L. donovani* promastigotes (100X)

which can multiply three to six times. It is a rapid method of drug screening and requires very less time ease of maintenance in cell suspension form in vitro. But this flagellated promastigote stage is not present in the host, and thus it is not an appropriate target for anti-leishmanial drugs. Drug screening on promastigotes at 26 °C is of no or little value because in vivo temperature (37 °C) and the temperature at which the promastigotes are growing are different. Promastigotes can survive at 37 °C but there is no multiplication. The main limitation for this model is that the promastigote stage data do not correlate with that of amastigote stage data (Suman Gupta and Nishi 2011) (Fig. 5.3).

Axenic Amastigotes as Drug Screening Targets Axenic amastigotes permit in vitro primary screening of a large number of compounds in lesser duration and in an easy manner, very much like promastigotes. It represents the in vivo situation because this parasitic stage exists in hosts. Axenic amastigotes were maintained in Schneider's *Drosophila* medium supplemented with 20% FBS and 1% of penicillin streptomycin solution with acidic pH of 5.5 and are incubated at 32 °C. Amastigotes (2×10^5 cells/ml) are seeded and allowed to grow and multiply in the presence or absence of test compounds for 90 h. The number of amastigotes is calculated by using a haemocytometer and compared with that of controls, which can grow four to five times of the starting concentration (Callahan et al. 1997). High-throughput screening for new anti-leishmanial drugs by using luciferase gene expressing DNA-transformed *L. infantum* axenic amastigotes has also been developed. Metabolic processes of axenic amastigotes differ from that of intracellular amastigotes Sereno et al. 2001). Drugs are tested directly on amastigote stage, and the lack of the interplay of host cell-mediated effects (macrophages, phagolysosome formation and drug-induced toxic effects on host system) is the main limitation of this model.

Intracellular Amastigotes as Drug Screening Targets It is the most popular and widely used reliable method for new anti-leishmanial drug screening. In this method, amastigotes are allowed to infect cultured macrophages, and generally BALB/c mouse peritoneal macrophages (primary macrophages), J774A.1 macrophage cell-line (BALB/c mouse origin) and human monocyte transformed macrophage cell lines (THP-1, U-937, and HL-60) are used as the host cells. Macrophages are infected with promastigotes (multiplicity of infection 1:10 ratio), and after 2 h of incubation, extracellular (non-phagocytosed) promastigotes are removed and incubated at 37 °C in 5% CO_2 environment along with different concentrations of test and standard drugs. After 72–96 h of incubation, the activity of the test compound is determined by microscopic observation of the number of amastigotes/100 macrophages, and % inhibition is determined (Suman Gupta and Nishi 2011) (Figs. 5.4, 5.5 and 5.6).

$$\%\text{Inhibition} = 100 - \left(\text{AT} \times 100 / \text{AC}\right)$$

AT – average number of amastigotes/100 macrophages in treated
AC – average number of amastigotes/100 macrophages in control

These differentiated non-dividing macrophages support the parasite division, and thus can be useful in the screening of new anti-leishmanial compounds. In vitro intramacrophage amastigote assay is a useful method for monitoring clinical resistance of leishmania parasites. New extension methods have been developed for this intramacrophage amastigote assay by applying quantitative real-time PCR, which can accurately determine the parasite DNA content in an amastigote-macrophage

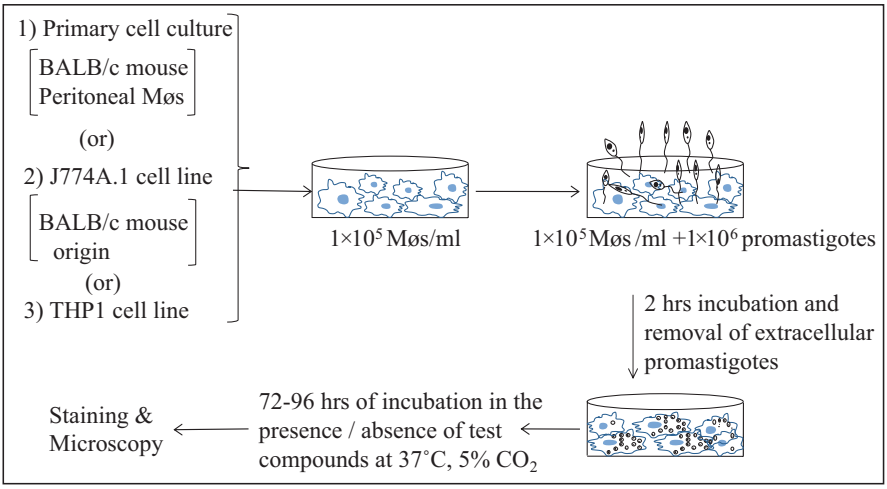

Fig. 5.4 Schematic representation of in vitro intramacrophage amastigote assay

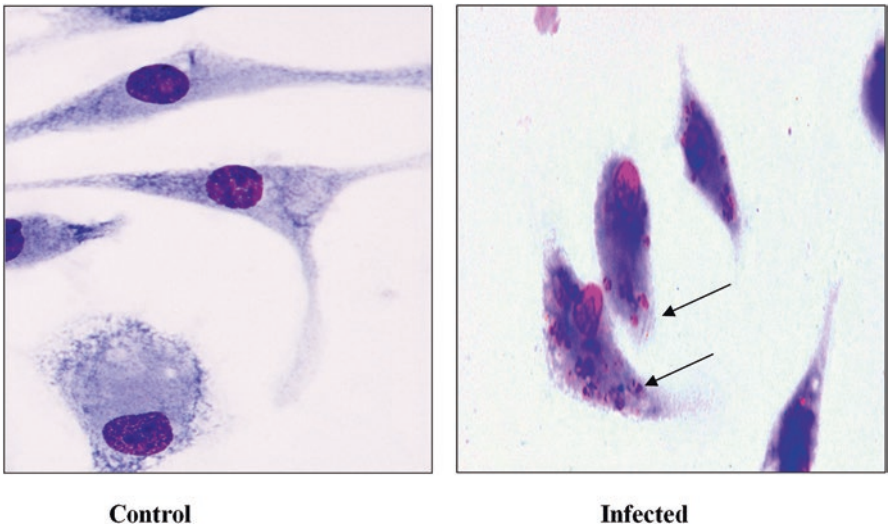

Fig. 5.5 Micro photographs of *L. donovani* amastigote-infected BALB/c mouse peritoneal macrophages (100X). Black arrows indicating *L. donovani* amastigotes

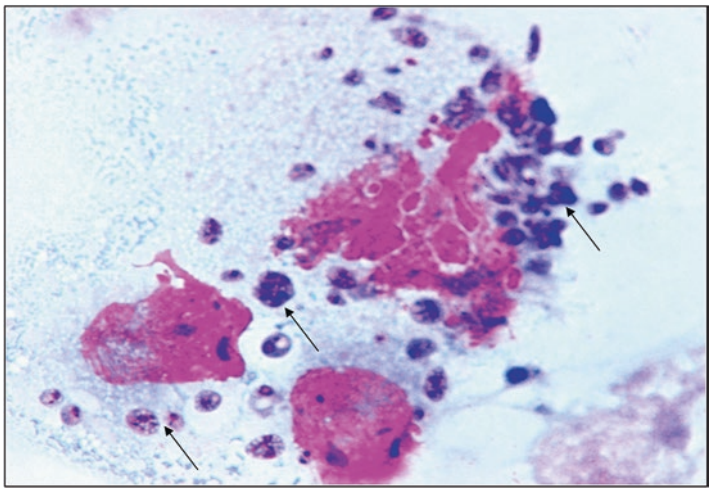

Fig. 5.6 Photomicrograph of *L. donovani* amastigote-infected J774A.1 macrophage (100X). Black arrows indicating *L. donovani* amastigotes

model. By using qPCR to this assay, we can overcome the difficulties and time-consuming microscopic counting which is involved in classical method (Gomes et al. 2012). Colorimetric assays have also been developed which can quantify the growth of intracellular amastigotes.

5.10 Reporter Gene Assays as Screening Models

Reporter genes generally encode a gene product that is a readily quantifiable phenotype and is easily distinguishable over endogenous cellular background. Based on their expression and existence, reporter genes can be classified as intracellular [chloramphenicol acetyltransferase (CAT), β-galactosidase, green fluorescent protein (GFP), firefly and bacterial luciferase and glucuronidase)] and extracellular reporter genes [(human growth hormone (HGH) and secreted alkaline phosphatase (SEAP)]. Reporter gene assays are advantageous as compared to conventional and currently available anti-leishmanial drug screening methods which are fraught with several problems like they are labour-intensive and time-consuming and lack automation (Dube et al. 2009). Genetically engineered recombinant leishmania parasites carrying reporter genes like GFP, CAT, β-galactosidase, firefly luciferase and alkaline phosphatases are mainly used reporter genes and facilitate monitoring of intracellular parasites. The main disadvantage of this method is the development of cross-resistance. In a high-throughput high-content intracellular *L. donovani*, assay in a 384-well plate has screened nearly 15,659 different compounds and has been proved better than the axenic amastigote screening method (De Rycker et al. 2013).

5.10.1 Green Fluorescent Protein (GFP) Assay

GFP, a bioluminescent protein, has been initially described as the green protein isolated from jelly fish *Aequorea victoria* (Chalfie 1995). Transfection of GFP in parasites like *Plasmodium* and *Leishmania* has been developed and used for drug-developing screening methods. Transgenic leishmania promastigotes which express GFP from episomal pXG vectors have proved the usefulness of GFP as a marker in the transfected leishmania (Ha et al. 1996). GFP is a cytoplasmic protein with low toxicity and has the possibility of continuous synthesis and ease of imaging and quantification. Introduction of GFP, as a marker in field strains of leishmania promastigotes by using leishmania expression vector pXG-GFP, has been developed, and in vitro anti-leishmanial compounds screening can be performed on these transgenic *L. donovani* promastigotes expressing GFP in their cytoplasm as a target in the cells. The relatively short duration of the screening experiments, possibility of automation, cost-effectiveness and the greater reliability are some of the main strengths of GFP assays and are more advantageous than classical drug susceptibility testing having the drawbacks of being labour-intensive and time-consuming and the requirement of macrophages (Singh and Dube 2004). GFP-tagged *L. donovani* promastigotes have been used for the observation of their developmental growth stages in the midgut of *Phlebotomus* vector easily as compared to non-tagged GFP (Guevara et al. 2001). *L. (Viannia) panamensis* promastigotes expressing GFP by transfection with p 6.5-egfp could retain its infection and are transformed into

amastigote stages in U937 and J774 cell lines, in vitro, and the intracellular parasites expressing GFP can be easily identified by fluorescence microscopy, and flow cytometry can be useful in high-throughput screening of new potential anti-leishmanial compounds (Munoz et al. 2009).

5.11 In Vivo Anti-leishmanial Drug Screening Models

To understand the host-parasite interaction, immunobiology and pathology of VL needs a proper animal model. This knowledge is useful for the synthesis and development of new anti-leishmanial compounds. BALB/c mice and Syrian golden hamsters are the commonly used primary drug testing laboratory animal models for VL. Canines (dogs) and primates (monkeys) are commonly used as secondary drug testing models in VL drug discovery and development processes.

5.11.1 Mouse Model

The mouse model of VL has been extensively used for the development of vaccines and other related immunotherapeutics. Inoculation of *L. major* promastigotes in mice permits us to identify the immunological mechanisms involved in resistance (C57/BL6 strain) and susceptibility (BALB/c strain) to leishmaniasis. In-bred mouse strains are relatively more susceptible to leishmania infection. The susceptibility observed in BALB/c mice is due to the development of Th2 cytokines (IL-4, IL-10 and TGF-β), which can deactivate macrophages and favour intracellular parasite growth. Resistance in C57/BL6 strain is due to the development of Th1-mediated cytokine IL-12 which can activate macrophages to release IFN-γ, stimulates inducible NOS to release NO and kills the leishmania parasites (Matte et al. 2000). The mouse host, especially BALB/c mice, is used for studying organ-specific immunology. In *L. donovani*-infected BALB/c mice, amastigotes are highly replicated in the liver in the first few weeks of infection. Genetically resistant mice have natural resistance-associated macrophage protein 1 (NRAMP 1) gene, which is mainly involved in macrophage activation that can kill leishmania parasite by nitric oxide-mediated mechanisms (Loria-Cervera and Andrade-Narvaez 2014). Intravenous inoculation of 1×10^7 promastigotes has been reported to induce infection in mice. In the early stages of infection, the parasite burden is more in the liver as compared to the spleen. For routine new anti-leishmanial drug screening studies, BALB/c mouse strain has been extensively used. BALB/c mice are infected with 2×10^7 *L. donovani* amastigotes, intravenously, on Day 0 and randomly divided into groups (each group contains five mice). Treatment is given on day +7 to day +11 (five consecutive days), and the mice are sacrificed on day +14. Liver tissue touch prints are prepared on glass slides and stained for parasitological observation.

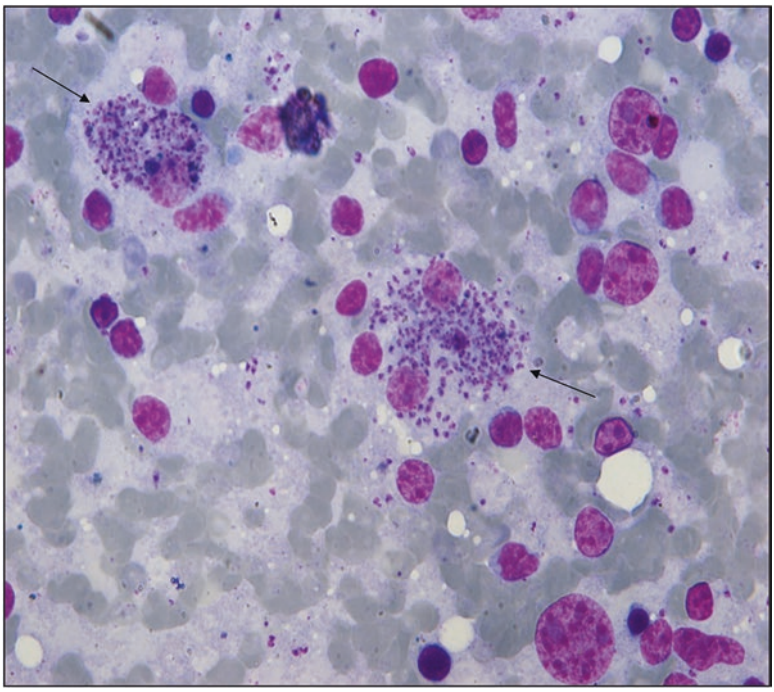

Fig. 5.7 Photomicrograph of *L. donovani*-infected BALB/c mouse liver tissue impression smears (100X). Black arrows indicate *L. donovani* amastigotes

The parasite burden is quantified as Leishman-Donovan Units (LDU; Suman Gupta and Nishi 2011) (Fig. 5.7).

$$\text{LDU} = \text{number of amastigotes} / 500 \text{host cell nuclei} \times \text{organ weight}(\text{mg})$$

5.12 Hamster Model

Syrian golden hamster (*Mesocricetus auratus*) is highly susceptible to infection by leishmania species like *L. donovani* and *L. infantum* and causes VL which absolutely resembles to human VL in clinical and pathological aspects like hepatosplenomegaly, pancytopenia, progressive cachexia, hypergammaglobulineamia, etc. It is extensively used as a screening model for new anti-leishmanial drug discovery (Nieto et al. 2011). An 8-day method for screening new anti-leishmanial drugs in golden hamster infected with 10^6–10^7 *L. donovani* amastigotes through intracardiac route has been developed for screening compounds in a short time (Stauber et al.

1958). *L. donovani*-infected hamsters are not able to control the replication of parasite due to failure of IFN-γ-mediated macrophage activation and decreased nitric oxide synthase-2 (NOS-2) activity due to defects in NOS-2 gene. NOS-2 is mainly involved in the production of nitric oxide (NO), a potent cytotoxic substance which kills the intracellular parasite. Leishmania-infected hamster macrophages do not generate NO, resulting in uncontrolled proliferation of intramacrophage parasites (Perez et al. 2006). The phagolysosome fusion has great implication in parasite survival, growth and multiplication in parasitophorous vacuole (Chang and Dwyer 1978). This knowledge is helpful in the chemotherapy of leishmaniasis. In VL patients haematopoiesis is severely affected which causes anaemia. Recently, it has been proved that induction of anaemia occurs due to the changes in erythropoiesis in the spleen and bone marrow of *L. donovani*-infected golden hamsters. Anaemia and leucopenia have been observed in 8 weeks of postinfection. Serum erythropoietin levels and BFU-E and CFU-E progenitor populations are greatly enhanced in the bone marrow and spleen of infected hamster (Lafuse et al. 2013). Because of the scarcity of immunological reagents, *L. donovani* -infected hamster model is not suitable for vaccination and immunotherapeutic studies. For overcoming the drawbacks of non-availability of immunological reagents for hamsters, recently, a new screening model has been developed. In this model, liver and splenic parasite burden was more in hamsters infected with 10^7 promastigotes, and not with 10^5 promasigotes, intracardially, after 155 days postinfection (Dea-Ayuela et al. 2007) (Figs. 5.8, 5.9, 5.10, 5.11 and 5.12).

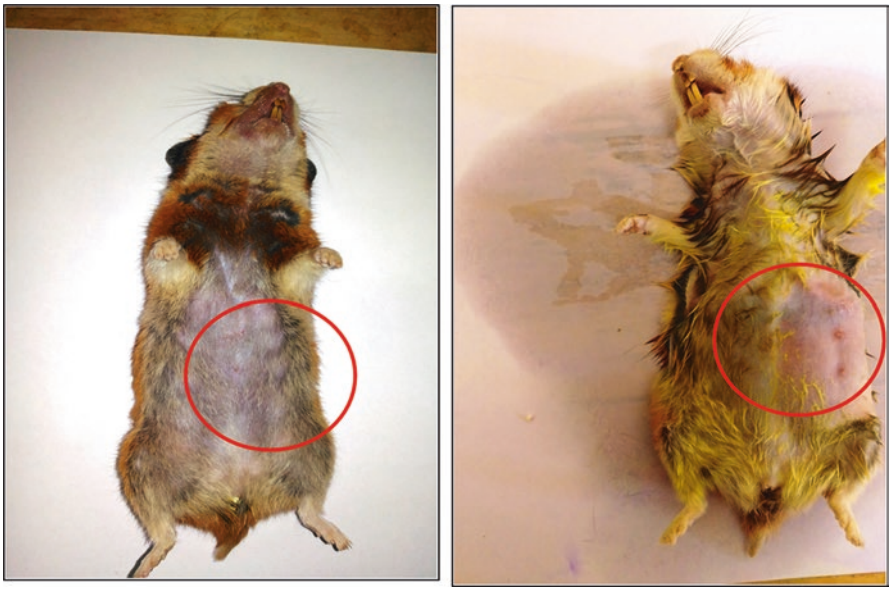

Fig. 5.8 Hepatosplenomegaly in *L. donovani*-infected hamster. The spleen and liver were enlarged in *L. donovani*-infected hamster compared to the control hamster

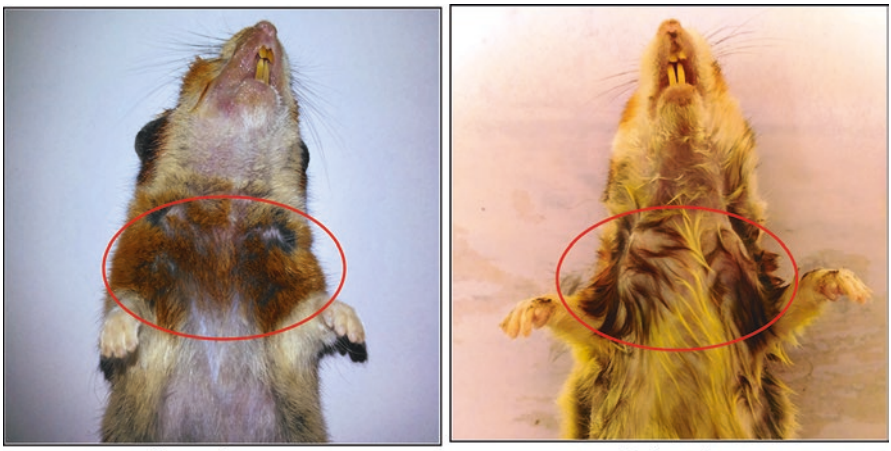

Fig. 5.9 Emaciation in *L. donovani*-infected hamster. Emaciation was clearly observed in *L. donovani*-infected hamster compared to the control hamster

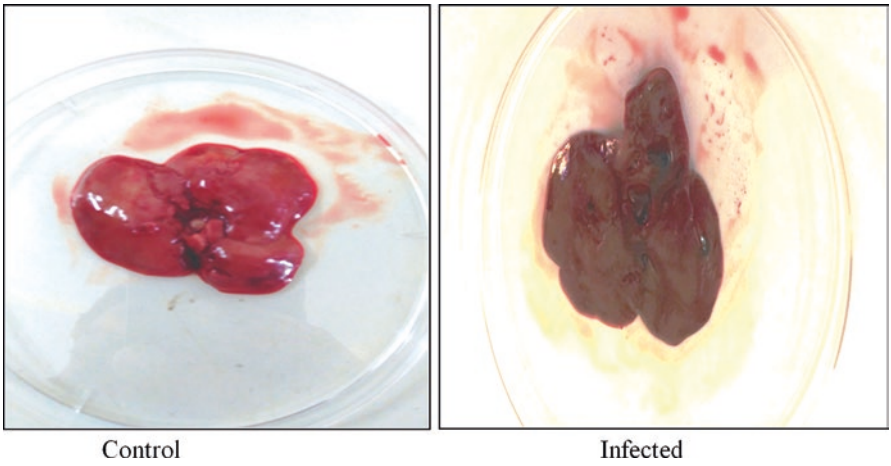

Fig. 5.10 *L. donovani*-infected hamster liver

5.13 Canine Model: A Secondary Drug Screening Model for VL

Canines are the best secondary drug screening models for VL. Drug metabolism and pharmacokinetic parameters in dogs, cats and monkeys are similar to human kinetic parameters. Mainly dog strains like stray, beagle and mongrel are more susceptible to VL infection and produce subclinical to fatal infection as like human VL. The

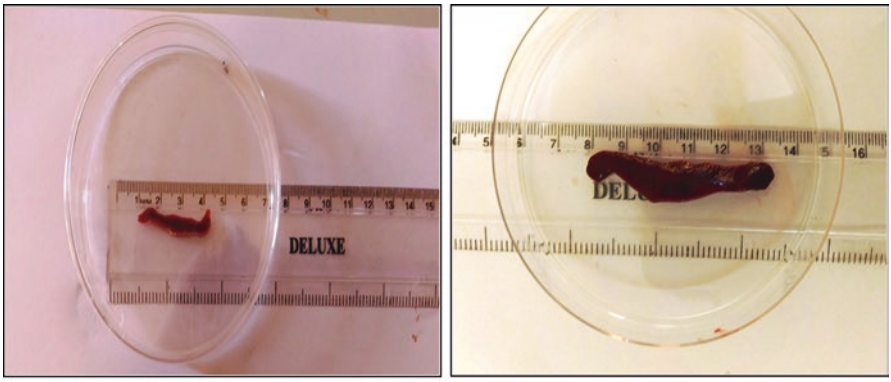

Fig. 5.11 *L. donovani*-infected hamster spleen

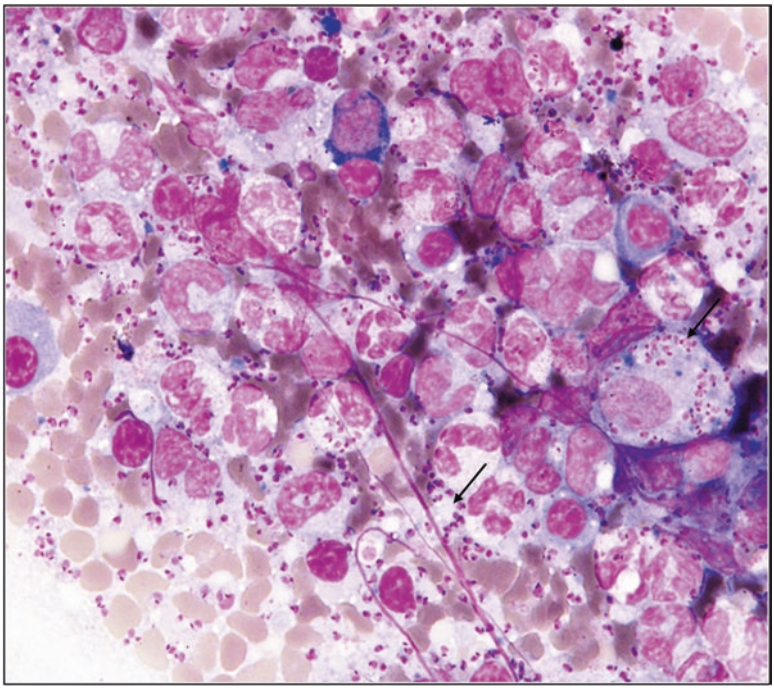

Fig. 5.12 Photomicrograph of *L. donovani*-infected hamster spleen tissue impression smears (100X). Black arrows indicate *L. donovani* amastigotes

dog is the major reservoir for *L. infantum* (in Middle East and Mediterranean region) and *L. chagasi* (in South America) (Loria-Cervera and Andrade-Narvaez 2014). Canine VL enhances the incidence of human VL in endemic regions where both the canine and human VL are prevalent, so development of vaccine against canine leishmania can reduce the incidence of human VL in both canine and VL endemic regions (Moreno and Alvar 2002). Anaemia, hypergammaglobulinaemia, fever, progressive weight loss with decreased appetite, swelling of lymph nodes, skin lesions and epistaxis (nose bleeding) are common pathological symptoms in canine leishmaniasis. In *L. infantum*-infected dogs, macrophages are known to be activated by IFN-γ and TNF-α to kill intracellular amastigotes through NO pathway; similar mechanism was observed in human VL. In *L. infantum*-infected dogs, m-RNA levels of IL-4 are greatly enhanced, and balanced production of Th1 and Th2 cytokines has been observed in infected dog spleen cells. Both CD4+ and CD8+ T-lymphocytes levels are to be diminished in *L. infantum*-infected dogs (Barbieri 2006). A transmission-blocking vaccine, FML vaccine (combination of FML antigen of *L. donovani* and saponin), has shown to protect against canine leishmaniasis (Saraiva et al. 2006). Because of the availability of huge immunological markers, the dog model of VL has become the best suitable experimental model for developing vaccine and immunotherapeutic agents for treating VL.

5.14 Primate (Monkey) Model: A Secondary Drug Screening Model for VL

Non-human primate models are phylogenetically close to human models. Physiology, pathology and immunology of non-human primates can mimic human VL. The monkey model is mainly useful for studying immunobiology of infection and host-parasite interactions. Non-human primate models like owl monkey (*Aotus trivirgatus*), squirrel monkey (*Saimiri sciureus*), marmoset (*Callithrix jacchus*), African green monkey (*Chlorocebus sabaeus*) and Indian langur monkey (*Presbytis entellus*) and vervet monkey (*Cercopithecus aethiops*) are commonly used preclinical models for anti-leishmanial drug screening studies (Olobo et al. 2001). Khartoum strain (WR378) of *L. donovani* amastigotes (3.25×10^7) that infected owl monkeys (total monkeys infected = 8) has shown the progressive weight loss, anaemia, hepatosplenomegaly, increased levels of serum hyperglobulinaemia, azotaemia and hyperalubinaemia, and high number of parasites have been observed in the liver, spleen, bone marrow and lymph nodes. Six owl monkeys died in 98 days of postinfection, and these findings support that owl monkeys are more susceptible to *L. donovani* infection and can be used as an animal model for VL drug discovery (Broderson et al. 1986). *L. infantum* (2×10^7 amastigotes/kg of body weight, *i.v.* route)-infected rhesus monkeys (*Macaca mulatta*) have shown clinical and immunopathological symptoms similar to human VL. Their findings suggested that rhesus monkey model is useful for preclinical screening of potential anti-leishmanial compounds and for the development of vaccine candidates for human VL (Porrozzi et al. 2006).

5.15 Conclusions

VL, a neglected tropical disease with high death toll every year, needs early diagnosis and proper treatment. Unfortunately, high cost, severe adverse effects of the currently available anti-leishmanials and emergence of leishmanial parasite resistant to pentavalent antimonials limit their clinical use. Development of highly sensitive and specific diagnostic methods for VL, HIV-VL and malaria-VL may be advantageous in leishmania disease control. Immunotherapy with cytokines (GM-CSF, IFN-γ and IL-12), both stand-alone and in combinations with current anti-leishmanials (immuno-chemotherapy), can emerge as a useful alternative therapy to halt the spread and consolidation of drug resistance. There is an urgent need for the discovery and development of new anti-leishmanial drugs which should be of low cost, require short-course therapy, have high oral bioavailability and are free from adverse effects, for the better control and management of VL. In vitro and in vivo drug screening methods are also available for testing of new anti-leishmanial compounds. In vitro intramacrophage amastigote method is a better reliable method which mimics human VL situation and is useful for the screening of potential new anti-leishmanials. In vivo animal models like rodent models, BALB/c mice, are useful in immunological studies, and Syrian golden hamsters are useful in chemotherapy studies. Higher models like canine model and non-human primate (monkey) models are useful secondary drug testing models for VL drug discovery and development.

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