Chapter 1

HISTORICAL DEVELOPMENT & CURRENT SCENARIO OF LEISHMANIASIS

1.1 Introduction

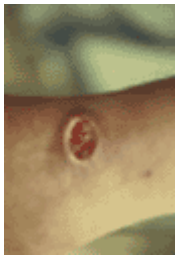
Parasitic diseases are always a burden for humanity. Protozoan parasites are responsible for several important diseases that threaten the lives of nearly one quarter of the human population worldwide. Protozoan parasite *Leishmania* is endemic in several parts of the world and remains a serious public health problem in numerous countries. Several species of this parasite are human pathogens and are responsible for one of three clinical forms of the disease, which include cutaneous, mucosal and visceral leishmaniasis (VL). Leishmania parasites were previously observed by David D. Cuningham in 1885 and Peter Brovsky in 1898. These parasites were mistaken for other protozoa, but later named as Helcosoma tropica by James Wright in 1903. Luhe in 1906 renamed it as *Leishmania tropica*. Similarly, the causative agent of VL was first named as *Pyroplasma donodvani* which was rechristened as *Leishmania donovani* by Ross in 1903 after its discoverers Leishman (1900) from London and Donovan (1903) from Madras who reported the organism independently (Herwaldt, 1999). There are more than 20 species of the genus, Leishmania that are pathogenic for humans and transmitted by an insect vector, the phlebotomine sand fly. The Leishmaniasis is now an emerging zoonosis in the United States (Enserink, 2000; McHugh et al., 2003; Rosypal et al., 2003) and US soldiers and peace keeping corps currently in the middle East are experiencing a large outbreak of leishmaniasis with more than 500 parasitologically confirmed cases (CDC, 2004).

1.1.1 Various forms of leishmaniasis

Leishmaniasis currently threatens 350 million men, women and children in 88 countries around the world. The leishmaniases are parasitic diseases with a wide range of clinical symptoms

cutaneous, mucocutaneous *and* ***visceral***.

1.1.1.1 Cutaneous forms

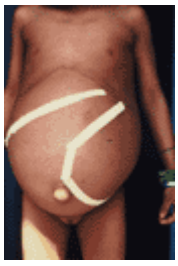


Cutaneous forms of the disease normally produce skin ulcers on the exposed parts of the body such as the face, arms and legs. The disease can produce a large number of lesions - sometimes up to 200 - causing serious disability and invariably leaving the patient permanently scarred, a stigma which can cause serious social prejudice.

1.1.1.2 Mucocutaneous forms

In mucocutaneous forms of leishmaniasis, lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues. These disabling and degrading forms of leishmaniasis can result in victims being humiliated and cast out from society.

1.1.1.3 Visceral forms



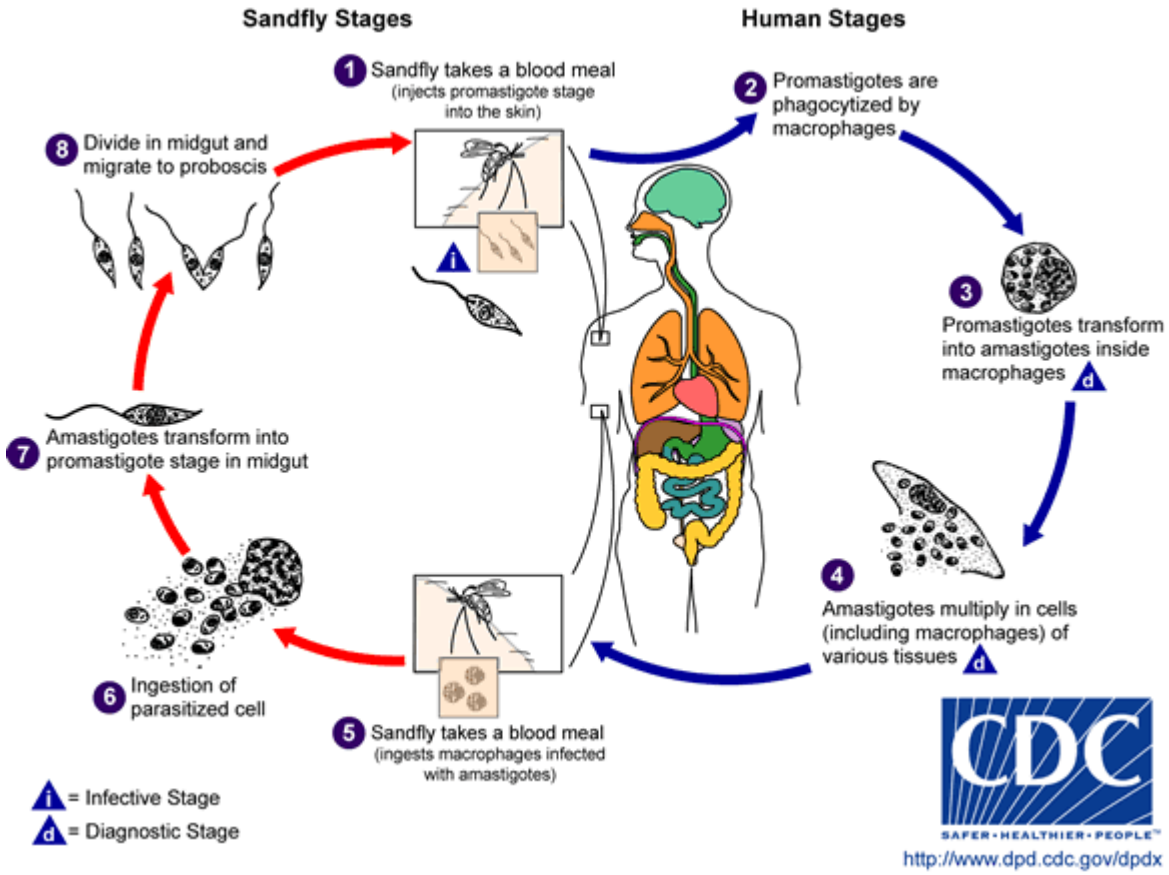
Visceral leishmaniasis - also known as kala azar - is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anaemia (occasionally serious). If left untreated, the fatality rate in developing countries can be as high as 100% within 2 years.

Human leishmaniasis is distributed worldwide, but mainly in the tropics and sub-tropics, with a prevalence of 12 million cases and an approximated incidence of 0.5 million cases of VL

and 1.5 million cases of CL. *Leishmania*sis, in particular *Leishmania* infantum infection which is also an important disease of dogs in Mediterranean countries and Brazil. These general aspects of *Leishmania*sis and overall control led strategies have been reviewed recently[1, 2].

1.2 Life Cycle of Leishmania parasite

Leishmaniasis is transmitted by the bite of infected female phlebotomine sandflies. The sandflies inject the infective stage (i.e., promastigotes) from their proboscis during blood meals (1). Promastigotes that reach the puncture wound are phagocytized by macrophages (2) and other types of mononuclear phagocytic cells. Progmastigotes transform in these cells into the tissue stage of the parasite (i.e., amastigotes) (3), which multiply by simple division and proceed to infect other mononuclear phagocytic cells (4). Parasite, host, and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. Sandflies become infected by ingesting infected cells during blood meals (5, 6). In sandflies, amastigotes transform into promastigotes, develop in the gut (7) (in the hindgut for leishmanial organisms in the *Viannia* subgenus; in the midgut for organisms in the *Leishmania* subgenus), and migrate to the proboscis (8).



Life Cycle of Leishmania Parasite

1.3 Amastigotes and Promastigotes

The intracellular amastigote is the mammalian stage of the parasite life cycle and is the target for chemotherapy. Although the amastigote is more difficult to culture than the promastigote stage, it is important that this relevant stage of the parasite is used for *in vitro* drug testing as the difference between the two stages is not just morphological; there are also important biochemical and molecular differences. These differences include glucose catabolism and utilization of fatty acids[3-6], nucleases[7], cysteine proteases[8], purine metabolism[9] protein phosphorylation[10], gene expression[11], and the surface membrane proteins, the metalloproteinase gp63 and lipophosphoglycan (LPG) [12]. These major biochemical differences provide some explanation for the difference in drug sensitivity between the two

stages, the standard example being the >100 fold lower *in vitro* sensitivity of extracellular promastigotes than intracellular amastigotes to the standard pentavalent antimonials used in clinical treatment. The recent establishment of axenic amastigote cultures has provided a new approach to enable the investigation on the basis of stage differences in drug sensitivity. It has been confirmed that amastigotes are intrinsically more sensitive to pentavalent antimonials and paromomycin than promastigotes but are less sensitive to amphotericin B[13-15]. However, there are differences in the results reported in these papers especially regarding the levels of antimonial sensitivity of intracellular as compared to extracellular amastigotes.

1.4 Species Sensitivity

There are at least 20 species of *Leishmania* of which 15 are known to be infective to humans. Different species are associated with different pathologies and forms of leishmaniasis. Although morphologically the species are considered to be similar, with the exception of parasites of the *L. mexicana* complex which have larger amastigotes, there is considerable biochemical and molecular variation between species and strains. Biochemical variation, in particular isoenzyme electrophoretic patterns, and molecular variation, including diffferences between kDNA and genomic fingerprinting[16,17], have been used to redefine the taxonomy of this genus. It is therefore perhaps not surprising that on the activity of antimonials, paromomycin, azoles and allopurinol that variation in species/strain sensitivity is mentioned as a key consideration in the use of these drugs in anti-leishmanial chemotherapy.

1.5 Chemotherapy of Leishmaniasis

A primary infection, if cured, usually leads to protection against further infection suggesting that it should be possible to generate a vaccine against *Leishmania*. Unfortunately, in practice there are as yet no effective vaccines against this parasite. Thus, control of the disease relies

primarily on chemotherapy. The main drug treatments recommended for both VL and CL were first introduced over 50 years ago. However, the position is changing and some new therapies are becoming available. Unfortunately, it now seems clear that the previous ambition to develop a single drug or drug formulation to be effective against all forms of leishmaniasis was too optimistic. Not only do Leishmania spp. differ intrinsically in their drug sensitivity, but also the visceral and cutaneous sites of infection impose differing pharmacokinetic requirements on the drugs to be used. Available drugs are limited in number and each has various shortcomings. The target for chemotherapy is the intracellular amastigote that survives and divides in tissue macrophages, whereby causing the disease. The amastigote resides within a parasitophorous vacuole, which resembles a secondary lysosome with a pH of 4.5–5.0. The acidic environment has implications for the amastigote's strategies for nutrient acquisition and ion homeostasis. These involve a variety of transporters that could mediate drug uptake or drug efflux, and so play a part in determining the parasite's susceptibility to chemotherapy. Current evidence, although far from comprehensive, suggests that different *Leishmania* spp. not only reside in different macrophage types, but also have differing adaptations that facilitate intracellular survival . Such species variations could account for some differences in drug susceptibility and make it imperative that appropriate laboratory models are used for assessing drug efficacy.

Table 1. Current treatment of Leishmaniasis

No	Therapy	Mode of action
1	Opium[18]	Enhances the cellular response in the host
2	Garlic	The garlic extracts inhibit the

		enzyme Acetyl Co-A and enhances the cellular immunity of the host.
3	Hypertonic Sodium Chloride Solution[19]	This is a local therapy and results in destruction of parasite due to osmotic effect
4	Zinc sulphate[20]	Enhances the response of helper type-1 T cells(T_H1) and enhances the activity of glutathione peroxidise and catalase in patients.
5	Cryotherapy[21]	Inhibition of multiplication of the parasite through intracellular freezing with a double freeze-thaw cycle using liquid nitrogen.
6	Thermotherapy[22]	It is administered using radiofrequency waves
7	Carbon dioxide Laser[23]	Induces specific thermoslysis of the infected tissue in the host
8	Dapsone	Inhibits the choline incorporation into lecithin of the

		cell membrane of the parasite
9	Azole reagents	Induces inhibition of P-450 mediated alpha-demethylation of Lanosterol, thereby blocking the ergosterol synthesis in parasite
10	Allopurinol	Induces depletion of the ATP pools and inhibition of the energy dependent metabolic pathways in the parasite
11	Pentoxifylline	Destruction of the parasite by the reduction of the macrophages' susceptibility towards vacuolation
12	Imiquimod	Activates local immune functions and stimulates the production of TH1 type response in the host.
13	Sitamaquine (Lepidene)	Not clear
14	Pentamidine	Inhibits polyamine biosynthesis in the parasite
15	Amphotericin B	Binds to the parasite membrane

		sterolsand alters its permeability selective to K^+ and Mg^+.
16	Rifampicin	Inhibits the DNA-dependent RNA polymerase in the parasite
17	Paromomycin(Aminosidine)	Acts upon the ribosomes, retards mictochondrial activities and inhibits sterol biosynthesis in the parasite.
18	Sodium Stibogluconate(Pentostam)	Induces apoptosis involving DNA fragmentation and externalization of phosphatidyl serine on the outer surface of the plasma membrane in the parasite.
19	Meglumine antimoniate(Glucantime)	Similar to pentostam
20	Miltefosine	Induces apoptosis in the parasite leading to cell death.
21	Interferons beta and gamma	Enhancement of leishmanicidal activity of human monocyte.

1.5.2 Conventional therapy against leishmaniasis

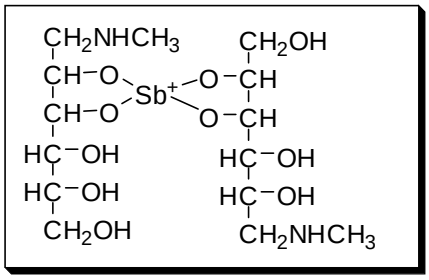
While conventional therapies i.e. pentavalent antimonials, amphotericin B and pentamidine (Figure 3) continue to play a major role, it is obvious that new drugs or strategies must circumvent limitations such as a long-term parenteral administration, toxicity (Table I), the high cost in endemic countries, and the emergence of resistance

Table 1 Adverse effects of conventional therapies against leishmaniasis.

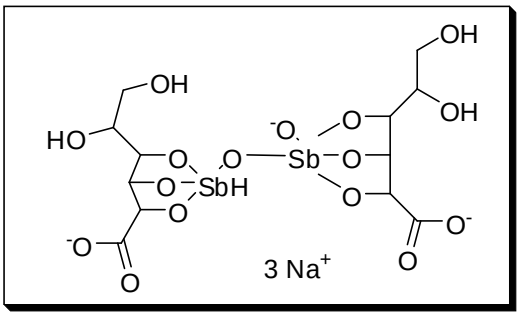
Drugs	**Adverse Effects**
Pentavalent Antimonials	arthralgia, myalgia, nausea, vomiting, abdominal pain, headache, rash, transaminase elevations, pancreatitis, anemia, leukopenia, thrombocytopenia, reversible renal insufficiency, and cardiotoxicity
Amphotericine B	fever, nausea, vomiting, malaise, anemia, phlebitis, hypokalemia, hypomagnesemia, and nephrotoxicity
Pentamidine	hypoglycemia followed by diabetes mellitus, hypotension (if administered too rapidly), nausea,

	vomiting, abdominal pain, and headache.
Miltefosine	vomiting, nausea, diarrhea

1.5.2.1 Pentavalent antimonials



Meglumine Antimoniate (Glucantime)



Sodium Stibogluconate (Pentostam™)

Figure 1

In a treatise published in Leipzig in 1604, antimony, introduced by Paracelsus as a general panacea in the 16th century, was acclaimed as one of the seven wonders of the world. Sometimes banned and often argued over for another three centuries, the modern era of usage began in 1905 when Plimmer and Thompson showed the activities of sodium and potassium tartrate against trypanosomes in rats and subsequently in the treatment of human trypanosomiasis in Africa. The first published records of use of these trivalent antimonials for treatment were by Macado and Vianna 1913 for CL and by di Cristina and Cariona in Sicily and Rogers in India in 1915 for VL[24]. Despite the spread of resistance and severe cardiac, hepatic, pancreatic and renal side effects, N-methylglucamine antimoniate (Glucantime) and sodium stibogluconate (Pentostam), (Figure 1) both pentavalent antimonials, remain the drugs of choice in most parts of the world[25]. The intracellular reduced trivalent form is the active derivative that comes about through the alteration in parasite bioenergetic pathways

and trypanothione inhibition[26, 27]. The recommendation for the use of pentavalent antimonials is administration by intramuscular injections of 20 mg Sbv/kg/day up to a maximum of 1275 mg over 20 or 30 days. In the case of old-world cutaneous leishmaniasis, there is no significant difference between the intralesional and intramuscular route[28]. For these drugs, the cure rate is generally high (85–95%), except in Bihar-India where 60% of patients with VL are now unresponsive[29], as in Iran for *L. tropica* infected patients (CL)[30] and in Peru for MCL[31].

1.5.2.1.1 Pharmacokinetics, Dosing Regimens and Delivery Systems

Pentavalent antimonials are absorbed quickly and excreted rapidly from the body (half life of approximately 2 hours), the remainder following around 76 hours later[32]. Despite the differences between *Leishmania* species and their clinical presentation, the recommended treatment regimen for antimonials is fairly uniform, namely 15-20mg SbV/kg per day for 21-28 days (40 days where resistance is reported), either intramuscularly or intravenously [33, 34]. Treatment regimens differ depending on the species involved, the health of the patient and the healthcare facilities and infrastructure available to the clinician. The long course of treatment allows anti-leishmanial levels of the drug to accumulate in the tissues, namely the liver and spleen. The lengthy dosing regimen of SbV treatment often causes side effects such as myalgia, pancreatitis, cardiac arrhythmia and hepatitis, leading to the reduction or cessation of treatment. Re-formulation of the drug reduced potential toxic side effects and increased activity of both liposomal and niosomal Pentostam against experimental CL and VL [32, 35, 36.] However, there is no commercial impetus to reformulate this old drug.

1.5.2.1.2 Species Sensitivity, Mechanism of Action and Resistance

Biochemical studies over the past two decades have indicated a number of potential targets for pentavalent antimonials; glycolysis[37]in particular inhibition of ADP phosphorylation[38],

DNA I topoisomerase[39, 40] and trypanothione[41, 42]. Molecular analysis *in vitro* established drug-resistant clones has suggested inhibition of fatty acid beta-oxidation[43] However, the precise mechanism of action of these drugs and the relevance of such studies will remain uncertain unless activities are related to pharmacologically achievable concentrations, remembering that the target cell is an intracellular parasite and there is a variation in strain/species sensitivity.

Recent research has started to define some ground rules. Firstly, it is important to be clear about the drug formulation being used. The demonstration, that the preservative m-chlorocrescol, present in the liquid formulation of Pentostam, has high antileishmanial activity, raises questions about all previous studies that used this commercial formulation in experimental studies rather than sodium stibogluconate powder[44]. As previous publications did not specify which form of the pentavalent antimonial was used, interpretation of past results is difficult. After 50 years, the complex chemistry of meglumine antimoniate (Glucantime) has been characterized and a major moiety [MW 507, has been identified[45]. However, despite efforts the exact nature of the complex polymeric carbohydrate sodium stibogluconate and its molecular weight has not been determined[45, 46]. Roberts and Rainey helped to dispel some of these worries, showing that 12 anion exchange fractions of Pentostam (after the m-chlorocresol had been removed) had almost equivalent antileishmanial activity. What did they not dispel were other concerns about variability and stability of pentavalent antimonial solutions, especially as batches with higher osmolarity are associated with reduced efficacy and increased toxicity [47]. Further concern about content was raised in 1995 by Franco *et al*, [48] who showed that batches of Glucantime contained between 10 to 15% trivalent antimony. Trivalent antimony is more toxic to *Leishmania* in both orders of magnitude and 10- fold more toxic to humans than the pentavalent form[49]. It is important to remember the differential *in vitro* sensitivity of promastigotes and amastigotes to

pentavalent antimonials, in comparison to trivalent antimonials. Until recently it was unclear whether this difference, with promastigotes insensitive to concentrations of 1000 mg Sb (V)/ml compared to amastigotes with an IC_{50} value of 10 mg Sb(V)/ml, was due in part to the influence of the macrophage host cell. The development of systems to culture axenic amastigotes has helped to partially clarify the situation. Using *L.mexicana* Callahan *et al.* (1997)[50]showed that axenic amastigotes had the same range of sensitivity as intra-macrophage amastigotes to both sodium stibogluconate and meglumine antimoniate, with IC_{50} values in the range of 30-50mg Sb (V)/ml (value for promastigotes >10,000 mg Sb (V)/ml). However, Ephros *et al.* (1997)[51] found *L.donovani* axenic amastigotes to be only 4-fold more sensitive than promastigotes, and Sereno and Lemesre (1997)[52] found *L.infantum*, *L.amazonensis* and *L.mexicana* axenic amastigotes to be only 2-fold more sensitive than promastigotes to pentavalent antimony. Both studies used formulations containing 0.1% m-chlorocrescol. In addition, the IC_{50} values for axenic amastigotes reported by Sereno and Lemesre (1997)[52] are about 10-fold higher than would be expected for intracellular amastigotes. Although these results confirm that amastigotes have a greater intrinsic sensitivity to pentavalent antimonials than promastigotes, they also confirm the caution necessary with reference to formulations, species and systems used. Drug uptake could contribute to the differential activity and early studies indicated that amastigotes have a superior ability to concentrate drug than promastigotes[53, 54]. However, when comparing promastigotes with intra-macrophage amastigotes, similar concentrations of antimony were determined in the parasite forms following incubation at (the very different) IC_{50} concentrations. Some resolution to the difference between amastigote and promastigote sensitivity has recently been described. They showed that axenic *L. donovani* amastigotes were able to metabolise Sb (V) to Sb (lll) more effectively than promastigotes although the mechanism was not identified. A third factor that influences the activity of antimonials is the

interaction with the host macrophage, either through accumulation or metabolism. In a J774 macrophage-*L.panamensis* model, Pentostam was poorly accumulated, to only 33% of the concentration in the external medium, after a 4-hour incubation period, in contrast to trivalent antimonials which were concentrated from the medium. However, there was evidence that macrophages could retain pentavalent antimony. Importantly, considering the nature of the parasitophorous vacuole, activity was maintained at low pH in keeping with previous observations[55]. Metabolism of penta- to trivalent antimonials by macrophages has recently been described involving host cell glutathione[56]. It would be of interest to see these studies repeated in different macrophage populations, bone marrow macrophages for example, and with cells at different stages of activation. Murray demonstrated that macrophage activation had a significant effect on Sbv accumulation and intracellular parasite killing[57]. There has also been much confusion about the nature of resistance to antimonials. *In vitro* studies on clinical isolates have shown there to be a wide variation in strain and species sensitivity to pentavalent antimonials, in amastigotes (in the macrophage model)[58, 59] in promastigotes[60-62] and in axenic amastigotes cultures. In repeated studies *L.major* amastigotes in murine macrophages are >3 fold less sensitive to sodium stibogluconate than *L.donovani* amastigotes. There is some evidence of the relevance of these *in vitro* studies to clinical treatment. In controlled studies *L.braziliensis* was significantly more sensitive to Pentostam than *L.mexicana*[63] and *L. major* was unresponsive to antimonial therapy[64]. Recent studies in India have demonstrated that acquired resistance to pentavalent antimonials is already making an impact on the treatment of visceral leishmaniasis. In the major focus of VL in Bihar, India 30-60% cases do not respond to drug treatment[65, 66]. Interestingly resistance is not present in an adjacent VL focus south of the Ganges. *L.donovani* parasites from patients in Bihar who did respond to Pentostam were found to be three-fold more sensitive than parasites from patients who did not respond to Pentostam using the *in vitro* amastigote –

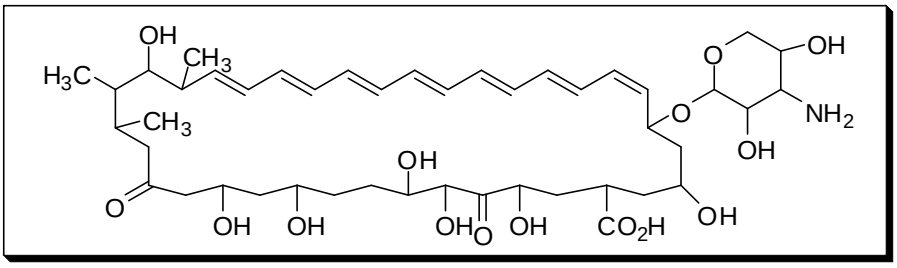
macrophage assay. There was no difference in the sensitivity of isolates when the promastigote assay was used[67]. An earlier report using VL isolates from Sudan also showed that clinical response to Pentostam was reflected in the amastigote-macrophage model but not in promastigotes[68]. Monitoring of antimonial resistance is a crucial issue in the endemic anthroponotic focus in India. It is unlikely that acquired antimony resistance will have an impact elsewhere as most leishmaniasis is zoonotic. However, it is worth noting that the significant decreases in the drug sensitivity of parasites taken before and after treatment with Glucantime have been observed in *L.infantum* infected patients in France and in *L. infantum* infected dogs before and after Glucantime treatment[69] Current evidence suggests that the mechanism of acquired resistance to pentavalent antimonials in *Leishmania* is dependent upon transformation of pentavalent compound to trivalent compound, formation of a conjugate with an elevated level of intracellular thiol (for example trypanothione) by an unidentified conjugase/transferase, and extrusion by elevated levels of ABC transporters[70-72]. This complex process has been determined *in vitro* using promastigotes. The recent description of Sb (V) to Sb (lll) metabolism adds another component that might have more importance in amastigotes.

1.5.2.1.3 Interactions with the Immune Response

Pentavalent antimonials have never been regarded as exerting an effect solely due to direct cytotoxic action. Schmidt and Peter[73] mention a moderate role for antimonials in the paradigm "the reticulo-endothelial system (i.e. its stimulation by drugs etc.) is of importance for the cure". Studies of murine VL infections (BALB/c – *L. donovani*) have established that an intact T-cell population, more specifically Th1, is required for Sb (V) to have a curative

anti-leishmanial effect[73-75]. The drug itself is leishmanicidal *in vitro* and *in vivo*, however complete cure, *in vivo*, is not achieved without Th1 input. Clinical observations support this – patients co-infected with VL/HIV respond poorly to treatment. After an initial response these patients frequently relapse and require alternative treatment. Dermotropic infections in men usually selfcure. This can take from 3 months to 3 years depending on the species of *Leishmania* involved. In such cases antimonial treatment augments the host's immune response to rapidly resolve the infection. Exceptional cases include DCL where in the absence of a cell mediated response antimonials prove to be ineffective[76].

1.5.2.2 Amphotericin B



Amphotericin B

Figure 2

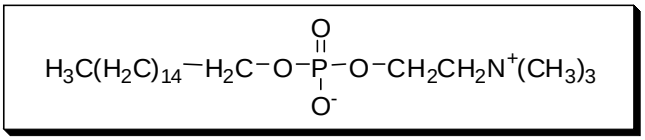
Another first line drug is amphotericin B (Fungizonew) (Figure 2) a macrolide polyene, characterised by hydrophilic polyhydroxyl and hydrophobic polyene aspects. Amphotericin B binds to membrane ergosterol leading to the formation of pores, major constituent efflux and,finally, parasite cell lysis. Intravenous infusion (7–20mg/kg up to 20 days) of amphotericin B is an alternative treatment in all the regions where antimonial resistance has been reported. This drug is characterized by infusion related side effects and renal toxicity[77]. Lipid based formulations such as liposomal amphotericin B (Ambisome) reduce the impairment of renal function by up to 50% with a therapeutic schedule of 6–21mg (LV) or 2–

3mg (ML)/kg for 20 days[78,79]. Despite its great efficiency, the prohibitive cost of liposomal amphotericin B limits its use in developed countries. Pentamidine isethionate, although used less frequently, diamine pentamidine has become of special interest in CL caused by L. guyanensis, where intralesional injections have been more efficient than with pentavalent antimonials[80]. Major side effects include hypotension, diabetes mellitus and renal impairment. Antileishmanial activity is based on the inhibition of polyamine biosynthesis and the disruption of mitochondrial membrane potential[81].

1.5.2.2.2 Mechanisms of Action and Resistance

The toxicity of amphotericin B is based upon the affinity of this polyene antibiotic to bind to sterols and to form aqueous pores in the membranes of cells[82]. Amphotericin B binds strongly to ergosterol, the predominant plasma-membrane sterol in fungi, *Leishmania* and *T. cruzi*. It has a slightly lower affinity for cholesterol, the sterol of mammalian cells. Toxic side effects result from this low therapeutic index. Various models have been suggested to illustrate the formation of aqueous pores through which ion leakage occurs, leading to cell death[82]. Against *Leishmania* promastigotes amphotericin B exerts concentration and time dependent effects. At low concentrations of amphotericin B (<0.1μM) cation channels are formed that collapse the membrane potential but are nonlytic, whereas at >0.1μM there is cation and anion influx through aqueous pores followed by osmotic changes and subsequently cell lysis[83]. Ketoconazole appears to reduce the sensitivity of *L. mexicana* promastigotes to amphotericin B through the depletion of desmethyl sterols[84]. Further studies on the ergosterol/episterol dependence of the mechanism of action of amphotericin B are required. Although resistance to amp B is rarely reported in eukaryotic

1.5.2.3 Miltefosine



Miltefosine

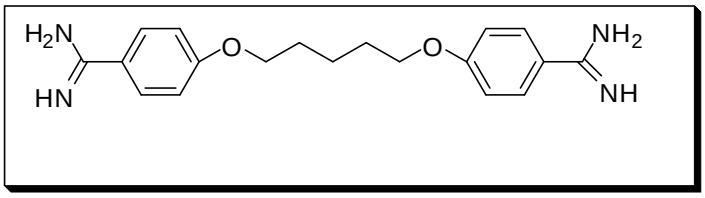
Figure 3

The most recently introduced drug in the armentarium is miltefosine (Impavidow) (Figure 3), an hexadecylphosphocholine derived from cancer therapy[85]. Miltefosine could alter glycosylphosphati

dylinositol (GPI) anchor synthesis, ether-lipid metabolism, signal transduction and alkyl-specific acyl-coenzyme-A acyl-transferase[86, 87]. With leishmaniasis, this first oral treatment has enabled us to attain high cure rates in Indian visceral leishmaniasis (95%) and Colombian cutaneous leishmaniasis (91%) when used at 100-150mg/day for 28 days[88, 89]. These clinical findings are in accordance with the laboratory results showing that L. *donovani* and L. panamensis are the most susceptible species[90, 91]. A phase IV trial conducted in 2006 in India demonstrated similar efficiency of miltefosine in field conditions[92]. Despite these encouraging reports, low cure rates observed in CL caused by L. braziliensis or L. major and transient cures followed by relapses in DCL or HIV/LV aco-infected patients could minimise its extended use as a monotherapy[93-95]. Miltefosine was first approved in India (2002), Germany (2004) and Colombia (2005). The antiprotozoal mechanisms of action of this class of drugs remain to be elucidated. There is some evidence, based upon studies of ether lipid metabolism in *L. donovani* promastigotes, that lipid biosynthetic enzymes represent a target[96]. Other studies have focussed on GPI anchor and ether lipid biosynthesis[97] but only found activity at high concentrations. Studies on mammalian cells have suggested that inhibition of

cell signalling pathways and induction of apoptosis are the mechanisms of action of ALPs; most of these pathways have yet to be sufficiently defined in protozoa.

1.5.2.4 Pentamidine



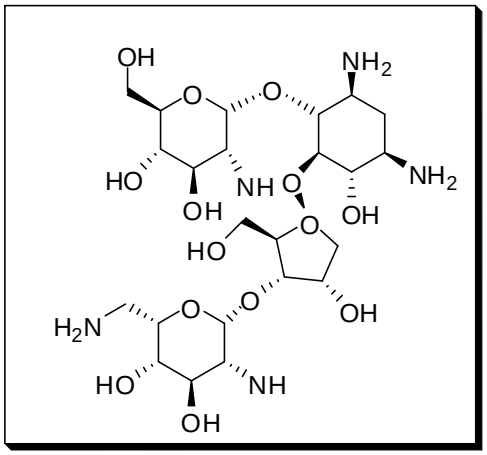
Pentamidine Isethionate

Figure 4

Pentamidine(Figure 4), an aromatic diamidine, as the isothionate salt (Pentacarinato) and previously as the methylsuphonate salt (Lomidineo), have been used as alternative treatments for both VL and CL since 1952 and as a primary treatment for *L. aethiopica* DCL. As a second line drug for antimony resistant cases, it has proved useful in India and Kenya[98]. Recently pentamidine was shown to be highly effective against CL in Colombia in a short course low dose regimen. Toxicity has always been a limitation on use with reports of hypoglycaemia, diabetes, nephrotoxicity, tachycardia, pain at site of injection . Pentamidine is still used for treatment of haemolymphatic stage of human African trypanosomiasis and, in combination with sulfamethoxazole, for *Pneumocytis carinii* pneumonitis (PCP) in AIDS patients .

1.5.3 Drugs in clinical trials

1.5.3.1 Paromomycin



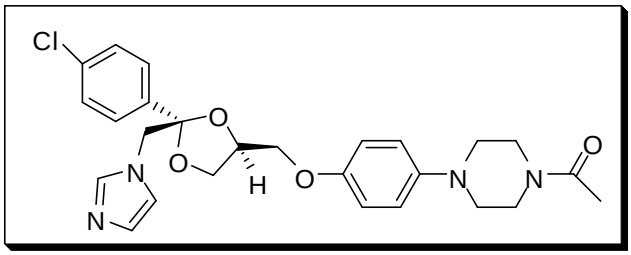
Paromomycin

Figure 5

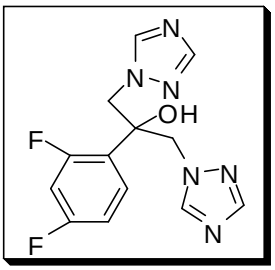
Paromomycin (Figure 5), an aminoglycoside antibiotic produced by *Streptomyces riomosus*, was developed in the 1960s as an oral therapy for intestinal protozoa. More recently, clinical formulations associating 15% paromomycin sulphate with 12% methylbenzethonium chloride (Leshcutan) or urea in paraffin have been investigated for topical treatment of cutaneous leishmaniasis[99-101]. Because of species- dependent efficiency and low tolerance (inflammation, burning sensation etc), a new formulation containing 15% paromomycin and 0.5% gentamycin in a complex base (WR 279396) was evaluated in a phase II trial in patients with L. panamensis cutaneous leishmaniasis[102]. No statistically significant difference was observed between cure rates after WR 279396 (61%) or placebo (55%) treatment. Recently, an Indian phase III trial was conducted to compare intramuscular paromomycin (15mg/kg/day) and amphotericin B in patients with L. *donovani* visceral leishmaniasis. This trial demonstrated that the two drugs were as effective at 6 months post-treatment with cure rates of 94.6% and 98.8%, respectively. Since August 2006, paramomycin IM has been approved in India as a new alternative for visceral leishmaniasis treatment. The mechanism of its action is unclear. In bacteria, this antibiotic binds to the A-site on the 16S RNA in the 30S sub-units of ribosomes giving rise to nonsense proteins through a misreading during protein

synthesis[103]. In *Leishmania*, paramomycin could interfere with RNA synthesis and membrane permeability[104]. The mechanism of action of aminoglyosides against bacteria are well known and relate to binding to the ribosome small subunit leading to mis-reading of mRNA[105, 106], and have been discussed as antiprotozoals in this context. Aminoglycoside resistance in bacteria is also well defined and can develop in three ways: (i) ribosomal changes, (ii) decrease in drug uptake, or (iii) metabolism of drug to an inactive form. The latter mechanism is considered to be of major clinical importance.

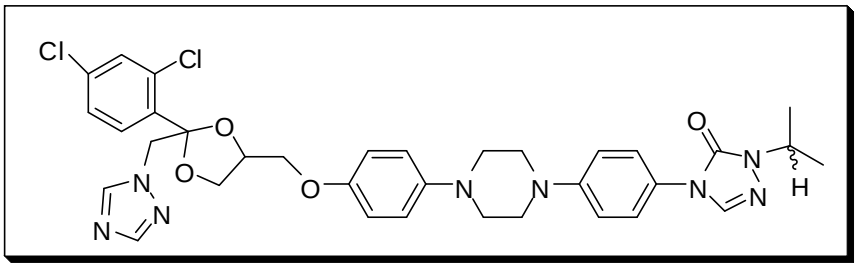
1.5.3.2 Azole derivatives



Ketoconazole



Fluconazole



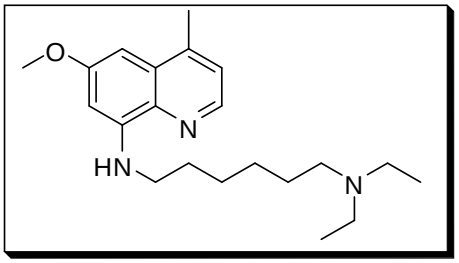
Itraconazole

Figure 6

The last example of development in new antiinfectious drugs is therapeutic switching also called “piggy-back therapy”. These include the azoles that were developed as antifungal drugs. Leishmania resembles fungi in synthesizing 24-substituted sterols such as ergosterol, whereas mammals have just cholesterol. Azoles, such as ketoconazole, inhibit 14a-demethylase, a key enzyme in this sterol biosynthesis pathway. Ketoconazole, itraconazole

and fluconazole(Figure 6) have undergone several trials for CL and VL with equivocal results. In one controlled trial, ketoconazole was found to have some activity against *L. mexicana,* but not against *L. braziliensis* infections. Some recent encouragement has been given by the oral activity of posoconazole in a *Leishmania amazonensis* experimental model.[106] As illustrated by the success of azole derivatives (fluconazole, voriconazole), due to the similarity in biochemical pathway, azoles are very effective in leishmaniasis therapy (ketoconazole, itraconazole)[107]. However, azoles have been the most controversial of these drugs, since the conclusions on efficiency come from few registered patients or poorlyconceived trials. Hence, a wide range of cure rates was reported for ketoconazole and itraconazole whereas promising results were obtained with fluconazole that reduced the time of healing of cutaneous lesions caused by *L. major*[108] or for posaconazole against experimental *L. amazonensis*[108].

1.5.3.3 Sitamaquine



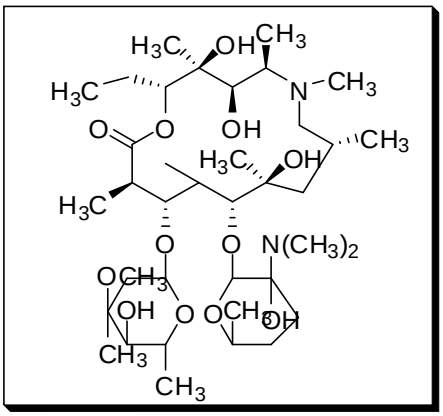
Sitamaquine

Figure 7

The intracellular targets of the 4-methyl-6-methoxy- 8-aminoquinoline, sitamaquine(Figure 7), are mitochondria and acidocalcisomes. The reason for the continued interest in this series of compounds is because one lepidine, WR6026 (8-{[6- (diethylamino)hexyl]amino}-6-methoxy-4-methylquinoline) a compound first synthesized in 1944, has reached clinical trials. This compound, 708 times more active than meglumine antimoniate against *L.*

donovani in hamsters[110], has been on clinical trial for VL in Kenya and Brazil.This compound has been used in clinical trials against new and old-world LV with 67–92% cure rates after oral delivery (1.7–2mg/kg/day) for 28 days. A phase II trial is going on to study safety and tolerance and to investigate the efficiency of a 21-day course of treatment in American visceral leishmaniasis. Further studies must be conducted in order to explore the clinical value in other forms of leishmaniasis.

1.5.3.4 Azithromycin



Azithromycin

Figure 8

Azithromycin (Figure 8), an azalide antibiotic has demonstrated activity against various protozoa i.e. *Toxoplasma gondii, Plasmodium spp*, *Cryptosporidium parvum*. The advantages of this macrolide are its high concentration in tissues, especially in macrophages, oral administration and safety. Its antiprotozoal action is due to the inhibition of protein synthesis but stimulation of phagocytosis, chemotaxis and its enhancement of immune response cannot be excluded[111-113]. Encouraging results were confirmed in American cutaneous leishmaniasis caused by L. braziliensis with high response rates (85%) at 60–120 days after varying periods of taking a 500–1000 mg daily dose[114,115]. However, trials conducted in endemic foci of *L.*

major in Iran, revealed no apparent role for azithromycin in old-world cutaneous leishmaniasis with 11.8%[116] and 10.3% [117]cure rates.

1.6 Resistance to antileishmanial drugs

Therapeutic failures resulting from treatment schedule modifications, such as sub-therapeutic doses or reduced treatment periods, are a typical situation in non-hospitalised patients. However variations in response to current antileishmanial drugs have indicated other aspects of resistance such as parasite intrinsic factors, drug pharmacokinetic and host immune status. The diversity of infection sites, which are specific to each clinical form, could contribute to the variations in efficiency of these drugs depending on their respective distribution in the host i.e. bone marrow, spleen, liver or dermis[118]. HIV/LV co-infected patients suffer from frequent relapses until the CD4 lymphocyte level increases, thanks to new antiretroviral therapy. Concerning the parasite counterpart, recent studies on the Leishmania genomic suggest a possible role of thiol metabolism and intracellular ABC transporter MRPAp in antimony resistance. Parasite resistance to amphotericin B could result from the replacement of the membrane ergosterol target by one of its precursor cholesta 5, 7, 24- trien-3β-ol after the alteration of transcripts of the C-24-D-sterol-methyltransferase. Resistance to pentamidine could be related to the loss of the drug transporter PRP1 in the plasmatic membrane or to lower accumulation in the mitochondrion thus facilitating its efflux. Data on the mechanism of miltefosine resistance come from laboratory manipulated strains showing that the overexpression of the P-glycoprotein efflux pump or the dysfunction of two proteins LdMT/LdRos3 that are necessary for its uptake, have been involved. Reports of lack of response to pentavalent antimony appeared in the 1970s and now this situation is wellrooted in Bihar-India and south-east Nepal. From the early 1980s to the 1990s, the cure rate after pentamidine administration decreased, demonstrating the increasing clinical resistance of

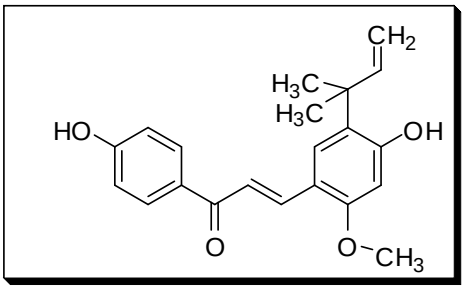
Leishmania to this drug. In the light of these considerations, the monitoring of this resistance phenomenon is of crucial importance as regards the medication available as first line treatment or recently introduced into an anthroponotic situation. Instead, the larger use of amphotericin B or miltefosine in endemic regions where Glucantime or pentamidine resistance is now established could rapidly generate wide resistance to case of *L. infantum* visceral leishmaniasis which as yet to be reported in an HIV patient.

1.7 Drug combination strategy

As in other infectious diseases, combination therapy should be one of the most important approaches to leishmaniasis. Drug combination could lead to reduced conventional treatment length, higher patient compliance and to the prevention of treatment failure, antileishmanial drug resistance or high toxicity. Furthermore, the combination of topical therapies could replace the need for parenteral injections of antimonials for LC. Combination therapies with antimonials; double-blind, randomised, controlled trial performed in Iran indicates that combined therapy using pentoxiffylline is more effective than Glucantime alone, with 81.3% and 51.6% complete improvement, respectively. In the same country, where *L. tropica* is endemic, Firooz et al. reported that a combined treatment with 5% imiquimod (Aldara), a topical immunomodulator, was no more effective than intramuscular antimonial alone. In this same region, imiquimod, however, showed a beneficial effect when meglumine antimonials were administered intralesionally[119]. When evaluated for American cutaneous leishmaniasis patients resistant to Glucantime, the combination demonstrated a cure rate of 72% in patients treated with glucantime plus imiquimod, while 35% of the patients receiving Glucantime plus vehicle cream healed at 3 months. Furthermore, this combination leads to faster healing and a better aesthetic aspect. Considering fatal Indian visceral leishmaniasis, treatment with paramomycin 18mg/kg on a daily basis combined with sodium stibogluconate 20 mg/kg for

21 days was statistically more effective than with sodium stibogluconate alone for producing final cure rates of 93.8% and 53.1%, respectively In addition, association with parenteral paromomycin allowed the reduction of the sodium stibogluconate treatment period (17 days versus 30 days). In the past, the combination of allopurinol plus sodium stibogluconate was investigated for few pentavalent resistant LV cases. Recently, in the special leishmaniasis recidivans clinical form, a chronic CL, highly resistant to current therapy, a combination with oral allopurinol (20 mg/kg for 30 days), was highly effective with 87.5% cure rates and only two relapses.

1.8 Plant products against Leishmania



Lichochalcone A

Figure 9

Although there are still no drugs present in the leishmaniasis armamentarium, interest in natural products must be maintained since chemical structure diversity could offer the basis for future drugs. Some studies concern the *in vitro* evaluations of purified compounds. Xanthinin, a xanthanolide from *Xanthium macrocarpum* harbouring an a-methylene-g-butyrolactone moiety, coumarin from *Cadophyllum brasiliense*, clerosterol from *Cassia fistula* and Jangambin from *Ocotea duckei* exhibited significant biological activities at promastigote or amastigote stages. A new taxoid from Taxus baccata, 10-deacethylbaccatin III, was reported to be highly active (IC_{50}, 70 nM) against the intracellular amastigote stage. Contrary to other taxoids, this activity does not seem to be related to the interference with

parasite tubulin. The *in vitro* reduction of the amastigote load was obtained after macrophage activation by propolis and garlic extracts. The latter could stimulate INFg and nitric oxide production resulting in the reduction of footpad lesions. In the mgroups of natural products evaluated, few demonstrated *in vivo* antileishmanial activity. Isopropylquinolines from Bolivian Galipea longiflora exhibit parasiticidal effect in experimental VL and CL. Two chalcone derivatives, licochalconeA[120] (Figure 9) from chinese Glycyrrhiza and more recently flavokavaı¨n B from Piper rusbyi and a triterpenoid saponin isolated from Maesa balansae (maesabalide III) are the most promising compounds. Protoberberine related to berberine, a chalcone from Berberis aristata, and maesabalide derivatives obtained through pharmacomodulation are under investigation .

1.9 New targets under investigation

During last few years, scientists have been convinced that rational drug design must be carried out in conjunction with investigations into Leishmania biology in order to better understand its particularities with the objective of defining new parasite targets for new therapeutic compounds. Specific enzymes that do not exist in mammalian cells are of particular interest as relevant targets. However, although there is structural homology with mammalian enzymes, those supporting a specific role in the parasite, could also offer fully functionally targets for antileishmanial therapy (Figure 10).

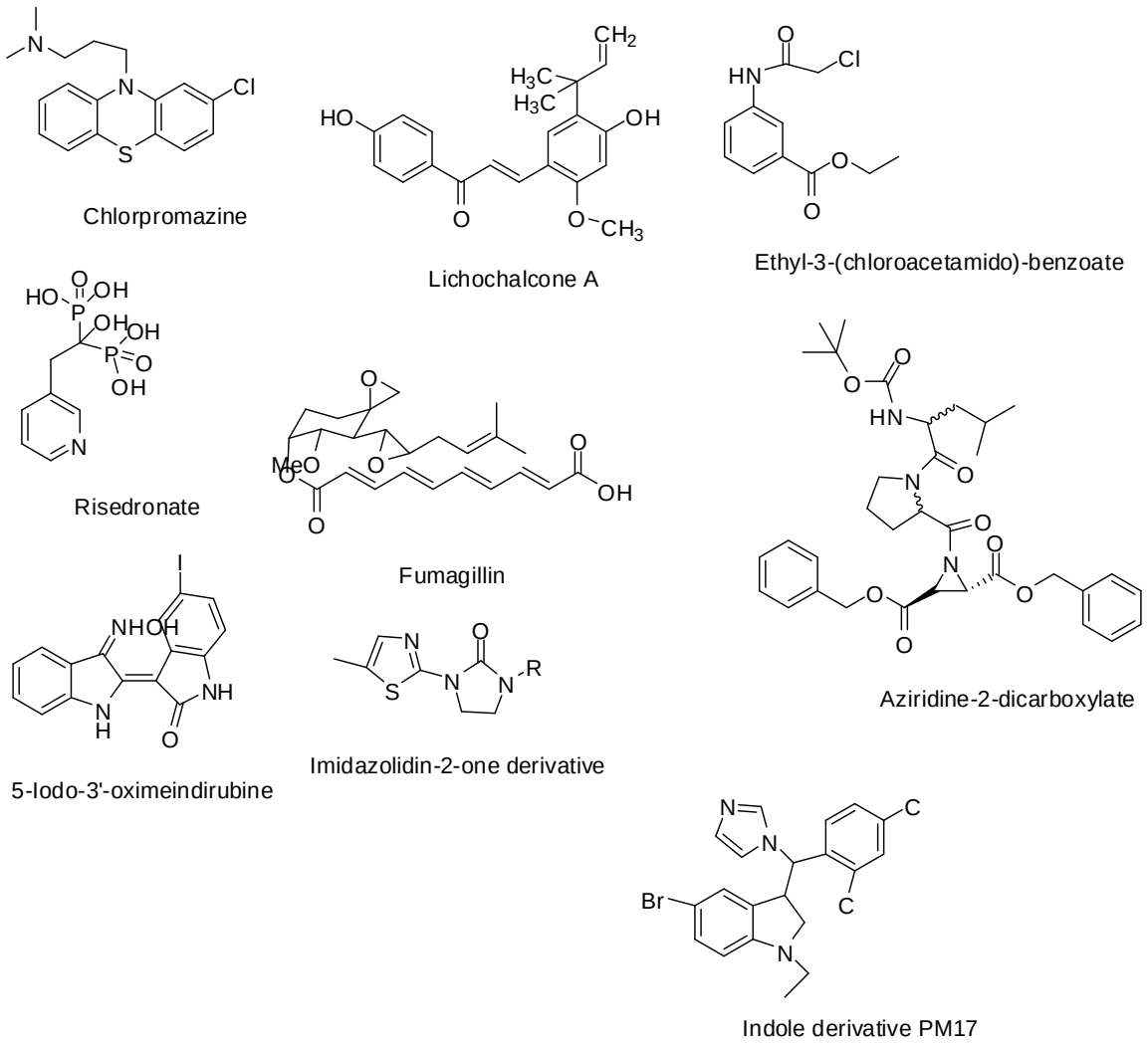


Figure 10

1.9.1 Trypanothione reductase/peroxidase

In Leishmania, glutathione is substituted by trypanothione (bis glutathionyl spermidin) to protect the cell against oxidants and xenobiotics. Specificity in the structure of trypanosomatid trypanothione reductase/ peroxidase active sites rationalise the design of parasite inhibitors[121]. Some 2-amino-4-chlorophenyl phenyl sulfide analogues of chlorpromazine inhibitors of trypanothione reductase from *Trypanosoma cruzi* were highly active against *Leishmania donovani*.

1.9.2 Topoisomerases

DNA topoisomerases are a class of enzyme involved in the regulation of DNA supercoiling and they are crucial during DNA transcription and replication. These enzymes are leishmanial targets for some new fluoroquinolones and pentamidine[122].

1.9.3 Sphingolipids synthesis

While the biosynthetic pathway for the formation of membrane sphingolipids of mammalian cells requires sphingomyeline synthase, in kinetoplastids inositol, phosphorylceramide synthase carries out the same role, indicating that it could be specifically targeted [123].

1.9.4 Fumarate reductase

In the Leishmania respiratory chain, succinate is converted into fumarate by fumarate reductase. As described before, this enzyme might be the specific target for the antileishmanial chalcones.

1.9.5 Microtubule associated protein (MAP2)

In the past, parasite microtubules have been identified as a potential target for therapy but the high toxicity of antimicrotubule drugs rapidly prevented their use in practice. Ethyl 3-(chloroacetamido)-benzoate, efficient on various Leishmania species, alters parasite microtubule organisation through interaction with a microtubule associated protein MAP2. Its *in vivo* activity was demonstrated in a *L. major* mice model by the important reduction of the parasite burden in the lymph node, spleen and liver.

1.9.6 Squalene synthase

In addition to lanosterol 14a-demethylase, squalene synthase involved in squalene formation by dimerisation of two molecules of farnesyl pyrophosphate, was identified as a new, valuable antileishmanial target[124]. Indeed, a 3-hydroxyquinuclidine (squalene synthase

inhibitor) and its derivatives induced Leishmania growth arrest after treatment with submicromolar concentrations. Recently, a recombinant *L. major* squalene synthase was produced which permitted the screening of selective inhibitors. Moreover, these compounds lead to major alterations in flagella, mitochondrial membranes and nuclear chromatin suggesting a possible apoptotic phenomenon. Located downstream from this enzyme, farnesyl pyrophosphate synthase was successfully targeted by biphosphonates such as risedronate.

1.9.7 Cysteine proteases

Cysteine proteases identified in the amastigote stage actively participate in differentiation, pathogenicity and virulence. Moreover, these enzymes are involved in the survival of Leishmania within the macrophage cells. The reduction of macrophage infection by cysteine protease inhibitors of aziridine-2,-dicarboxylate series could be mediated through the inhibition of parasite replication and the increase of nitric oxide production[125].

1.9.8 Methionine aminopeptidase 2 (MetAP-2)

MetAP-2 is a cellular metallo-exopeptidase that takes part in late hydrolysis of the initiator methionine of protein synthesis. A MetAP-2 inhibitor, fumagillin, blocked the replication of Leishmania donavani[126]. The three-dimensional structure of this enzyme has been modelled for Plasmodium falciparum showing difference in the binding pocket between parasite and human MetAP-2.

1.9.9 Protein kinases

A CDC2-related protein kinase encoding by the CRK3 gene is essential for controlling cell cycle progression at the G2/M-phase transition[127]. Included among the potential inhibitors of CRK3 cyclin-dependent kinase of Leishmania, indirubin derivatives exhibited growth arrest

and change in the DNA content. Evidence that PKC plays a critical role in the invasion process, is highlighted by a study demonstrating that pre-treatment of intact parasites by imidazolidinone compounds which inhibited PKC activity and the parasitehost cell invasion process. IL4 production. Indole derivatives have been reported as provoking the inhibition of interleukine-4 (IL-4) secretion. So, it was considered that pharmacomodulation associating azole and indole fragments in the same compound could be of great interest in leishmaniasis treatment. In the series of 3-(2-azolylbenzyl)indoles, one compound exhibited high *in vitro* and *in vivo* antileishmanial activity. Concerning the study on mechanism of action study, as anticipated, it was highlighted that it decreases ergosterol biosynthesis leading to membrane fungal cell alteration. Moreover it was proved that this imidazole antifungal agent induces a parasite burden-correlated decrease in IL-4 production, both in the splenocytes and the lymph node. The 3D-QSAR CoMSIA study offered a new model for further design of more promising inhibitors in the 3-(imidazol-1-ylmethyl)indole series.

1.10 Conclusions

Fewdrugs are available for treating *Leishmania* infections and the emergence of drug resistance is further complicating the control of leishmaniasis. A better understanding of resistance mechanisms and mechanism of action of drugs may point the way to more rational uses of drugs and drug combinations, which would help minimize resistance development and achieve more effective treatments. Combination chemotherapy is rapidly emerging as the norm for treating several parasitic infections; drug combinations have already been used empirically to successfully treat refractory isolates. Controlled clinical trials are in the planning stage for combination chemotherapy in *Leishmania* and may be required to protect the novel oral agents miltefosine and fluconazole from quickly becoming obsolete. Studies of resistance will also permit the identification of key intracellular targets and parasite defence mechanisms, which can then be exploited to rationally develop analogues of existing drugs

that would not be affected by the most common defences. For example, work *in vitro* and *in vivo* has shown that trypanothione biosynthesis inhibitors may sensitize resistant *Leishmania* cells to pentavalent antimony. Further studies are warranted to try to find a drug combination that could reverse resistance. A better understanding of drug resistance mechanisms in the field will also allow the development of new diagnostic assays, such as nucleic acid-based tests, that could rapidly detect a resistance gene. These could be useful to guide therapy and prevent the use of possibly ineffective and toxic agents. In this review, we have limited discussion to mechanisms of resistance to drugs that are useful in the clinical treatment of *Leishmania* infections. There are several other studies of resistance against experimental or model drugs that have highlighted the ability of *Leishmania* cells to overcome drug pressure and that have increased our understanding of the biology of the parasite. These studies have allowed the characterization of new resistance mechanisms and revealed new targets. Studies of resistance have permitted the integration of metabolic pathways in the resistance phenotype (Guimond et al., 2003), and were key in our understanding of gene amplification mechanisms and gene rearrangements in this parasite (Beverley, 1991; Grondin et al., 1996; K¨undig et al., 1999; Segovia, 1994). Finally, some of the initial vectors for gene transfection in *Leishmania* were derived from work on resistance (Kapler et al., 1990). The genome of *L. major* (http,//www.genedb.org) and of *L. infantum* (http,//www.sanger.ac.uk/projects/protozoa/) are completed and this should facilitate the discovery of novel targets and novel drugs that are required for treating *Leishmania* parasites. While there might be a disconnection between the rapid increase in our understanding of parasite biology and the discovery of novel drugs, there are tangible initiatives led by philanthropic or nonprofit organizations for the development of novel antiparasitic agents. The availability of transcriptomic and proteomic approaches have already led to the discovery of resistance mechanisms in this parasite more comprehensive work is likely to further

increase our understanding of resistance mechanism. Although pentavalent antimonials are still the first choice of drugs in most countries, several trials have undertaken the hunt for less toxic and less expensive drugs and for orally available treatment. The recent availability of oral miltefosine for visceral leishmaniasis has been the most significant development in the past few years. Nevertheless, the spectrum of resistance is always present, indicating the need for further research in order to assess, in greater depth, the efficiency and safety of drug combinations. Efforts to find new lead compounds and to identify new targets will also contribute to the fight against leishmanial diseases and the preparation of additional resources for the drug discovery pipeline.

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