

LONDON
SCHOOL of
HYGIENE
& TROPICAL
MEDICINE



LSHTM Research Online

Croft, SL; Seifert, K; Yardley, V; (2006) Current scenario of drug development for leishmaniasis. The Indian journal of medical research, 123 (3). pp. 399-410. ISSN 0971-5916
<https://researchonline.lshtm.ac.uk/id/eprint/11722>

Downloaded from: <http://researchonline.lshtm.ac.uk/11722/>

DOI:

Usage Guidelines:

Please refer to usage guidelines at <https://researchonline.lshtm.ac.uk/policies.html> or alternatively contact researchonline@lshtm.ac.uk.

Available under license: <http://creativecommons.org/licenses/by-nc-sa/2.5/>

<https://researchonline.lshtm.ac.uk>

Review Article

Indian J Med Res 123, March 2006, pp 399-410

Current scenario of drug development for leishmaniasis

Simon L. Croft, Karin Seifert* & Vanessa Yardley*

*Drugs for Neglected Diseases Initiative (DNDi), Geneva & *Department of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK*

Received May 13, 2005

Although three new drugs or drug formulations, liposomal amphotericin B (AmBisome), miltefosine and paromomycin should be available for the treatment of visceral leishmaniasis (VL) within the next year, they all suffer from limitations of either cost, specific toxicities or parenteral administration. As part of research to identify better treatments for VL and cutaneous leishmaniasis (CL), alternative and potentially cheaper formulations of amphotericin B, alkylphosphocholines other than miltefosine and improved formulations of paromomycin for CL have been identified. Other drugs or compounds that have demonstrated activity in experimental rodent models of infection include licochalcone derivatives, quinoline derivatives, bisphosphonates and a maesabalide; further chemistry based upon these leads is warranted. The process for discovery and development of new antileishmanials would also benefit from improved models, for example, transfected parasites, and non invasive methods of measuring parasite load in rodent models of infection.

Key words Amphotericin B - cutaneous leishmaniasis - first line drugs - miltefosine - toxicity - visceral leishmaniasis

The recommended drugs used for the treatment of both visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), the pentavalent antimonials, were first introduced 60 yr ago. Over the past decade alternative drugs or new formulations of other standard drugs have become available and registered for use in some countries, whilst other drugs are on clinical trial for both forms of the disease. Although the ambition to develop a single drug or drug formulation effective against all forms of leishmaniasis is unlikely to be fulfilled, the advances have been significant as the concept of choice for treatment is now real. The

problems of developing a single drug or formulation for all forms of leishmaniasis revolve around factors that include: (i) the visceral and cutaneous sites of infection impose differing pharmacokinetic requirements on the drugs to be used; and (ii) the intrinsic variation in drug sensitivity of the 17 *Leishmania* species known to infect humans. Other problems to be surmounted by new treatments for leishmaniasis are (iii) the need for drugs active in Bihar State, India where there is acquired resistance to the pentavalent antimonials¹; and (iv) increased efficacy in immunosuppressed patients, in particular due to

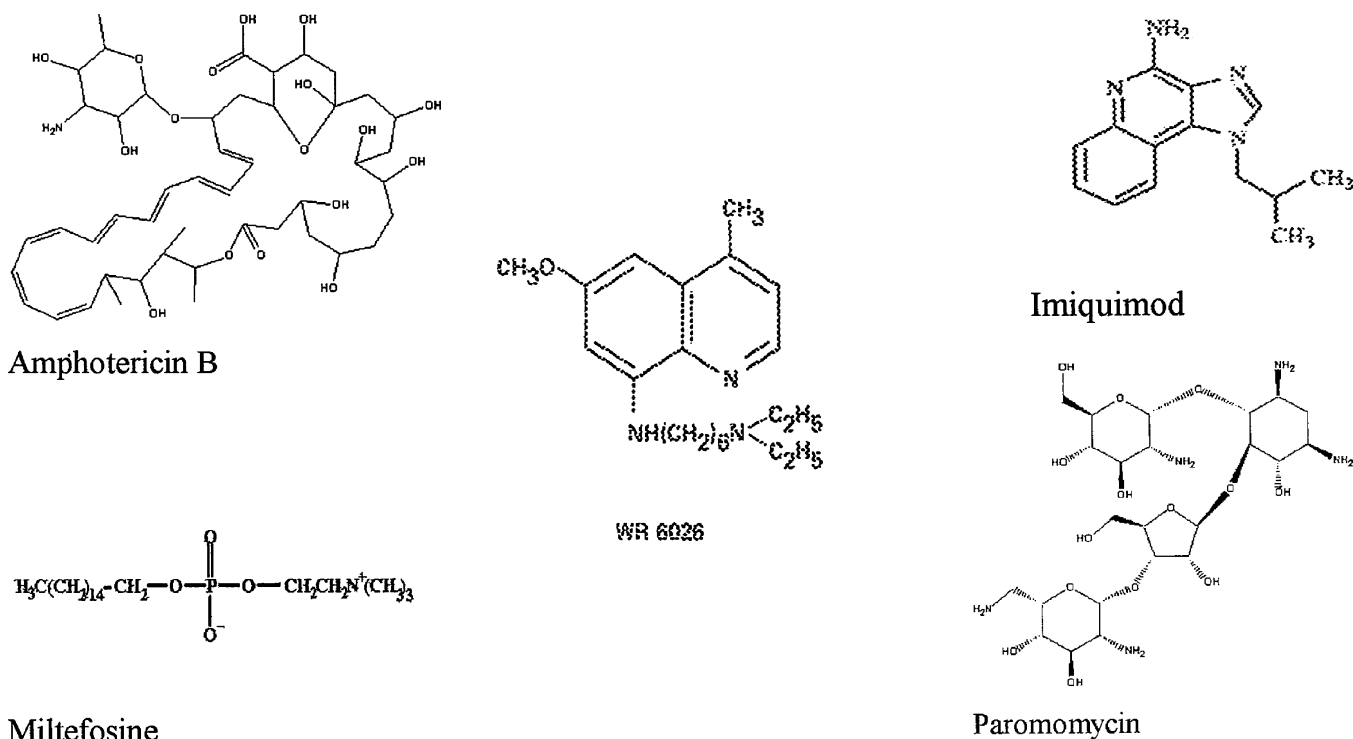


Fig. Structures of antileishmanial drugs on clinical trial.

HIV co-infection. In the latter case, where there is exacerbation of disease or emergence from latent infection, the depleted immune capability means that standard chemotherapy is frequently unsuccessful²⁻⁵.

This review will give an overview of (i) drugs that have been recently introduced and are still in phases of clinical trials or not widely registered, (ii) novel compounds and drugs that have shown activity in animal models and are worth considering for lead optimization; and (iii) specific methodologies involved in the discovery of new drugs.

Drugs in clinical development (Fig.)

Miltefosine: Miltefosine, initially developed as an anticancer drug, is the first effective oral treatment of VL and the latest antileishmanial drug to enter the market⁶. Its antileishmanial activity was initially discovered in the mid-1980s and efficacy demonstrated in a number of experimental models *in vitro* and *in vivo*⁷⁻⁹. These findings led to clinical trials and registration in India in March 2002 (Table)

for oral treatment of VL and in Colombia for CL in 2005.

There are concerns about teratogenicity and the long half-life of the drug, and that the latter might encourage the emergence of resistance¹⁰. It has been shown *in vitro* in laboratory studies on promastigotes that miltefosine resistant lines of *Leishmania donovani* can be selected¹¹ and resistance is related to two mutations on a transporter, the aminophospholipid translocase LdMT¹². Variation in species sensitivity, demonstrated *in vitro* with *L. donovani*, *L. aethiops*, *L. tropica*, *L. mexicana* and *L. panamensis* being more sensitive (EC₅₀ values between 2.63 and 10.63 μM) to miltefosine than *L. major* (EC₅₀ value 37.17 μM)¹³, is also a concern. The variation, also demonstrated in clinical isolates¹⁴, needs to be taken into account in drug use as it could contribute to different clinical outcomes in different regions as observed in a recent trial against CL in Colombia and Guatemala¹⁵. In Colombia, in regions where *Leishmania vianna panamensis* is common, the cure rates for miltefosine were 91 per cent

(40 of 44 patients), whereas in Guatemala (regions where *L.v.braziliensis* and *L. mexicana mexicana* are common) the cure rates achieved were only 53 per cent (20 of 38 patients) and lower than the historic antimony cure rates of >90 per cent.

Teratogenicity, potential of resistance development and a low therapeutic window pose limitations on miltefosine and it is desirable to optimize its structure with regard to activity and/or overcoming these limitations. Thus (SAR) studies have been carried out and a recent study explored the influence of cycloalkane rings in the lipid portion as well as headgroup modifications on activity towards *L. donovani* and *L. infantum* promastigotes and cytotoxicity towards THP-1 cells¹⁶. With regard to headgroup SAR, choline is preferred resulting in similar activity against both *Leishmania* strains, whereas the introduction of cycloalkylidene groups in the lipid portion results in enhanced activity, more pronounced against *L. infantum* than *L. donovani*. The mechanism of action of miltefosine is still not known. Several molecular targets in trypanosomatids have been suggested, including ether lipid metabolism, glycoposphatidylinositol (GPI) anchor biosynthesis, signal transduction and induction of apoptosis¹⁷⁻¹⁹, as well as inhibition of an alkyl-specific acyl-CoA acyltransferase in *L. mexicana*²⁰. Miltefosine also displayed marked activity against another trypanosomatid, *Trypanosoma cruzi*, with EC₅₀ values < 2 µM against intracellular amastigotes of the Y strain in murine macrophages⁹. Further studies on miltefosine of basic and preclinical nature are underway, including interaction studies for the potential use of drug combinations²¹.

Paromomycin: Paromomycin (PM), an aminoglycoside antibiotic, was originally identified as an antileishmanial in the 1960s and has been used in clinical trials for both VL and CL. Development of the parenteral formulation of PM, a drug with poor oral bioavailability, for VL has been slow, but phase III trials are currently ongoing in India under the aegis of the Institute of One World Health (www.iowh.org) and in East Africa managed by DNDi and partner institutes (www.dndi.org).

As with miltefosine, resistance to paromomycin could be induced in *L. donovani* promastigotes experimentally *in vitro*. The resistance was specific to PM and stable and its mechanism seems to be due to decreased drug uptake²². Ribosomes have been implicated as target²³ and inhibition of RNA synthesis followed by protein synthesis shown, along with induction of respiratory dysfunction²⁴. With PM moving into the field more studies on the mechanism of action and, more importantly, resistance are required. Variation in species sensitivity *in vitro* has also been demonstrated for PM with *L. braziliensis* and *L. mexicana* being less sensitive (EC₅₀ values between 38 and 39.4 µM) than *L. major*, *L. tropica* and *L. panamensis* (EC₅₀ values between 0.9 and 4.9 µM)²⁵. It is interesting to note the considerable differences between different isolates of *L. donovani* (EC₅₀ values ranging from 6.1 to 165.7 µM) demonstrated in the same study. Findings of this nature could have important implications in clinical outcome. For an Ethiopian strain (MHOM/ET/67/L82) of *L. donovani* marked synergy with sodium stibogluconate was demonstrated *in vitro*, to a lesser extent against an Indian strain (MHOM/IN/82/PATNA I). The interaction *in vivo* in BALB/c mice against MHOM/ET/67/L82 was additive. A combination of PM and sodium stibogluconate has been the subject of various clinical trials in Sudan and India^{26,27} but further studies to optimize the combination and define drug-drug interactions are required.

PM might also be a drug suitable for the topical treatment of CL. The report by El-On and colleagues in 1984²⁸ that a topical formulation containing 15 per cent PM and 12 per cent methyl benzethonium chloride (a skin-penetrating agent) was effective against experimental CL led to the clinical trials. One such trial demonstrated that 77 per cent were cured after 20 days treatment compared with 27 per cent cured in the placebo group²⁹. Other topical formulations with a lower skin irritancy have recently been on clinical trial, including one containing 15 per cent PM with 10 per cent urea and another containing 15 per cent PM with 0.5 per cent

gentamicin in a 10 surfactant vehicle (WR279 396) that cured 64 per cent of CL patients after 20 days treatment in Colombia³⁰. In an endemic area of Iran the 15 per cent PM/10 per cent urea showed no efficacy on cutaneous leishmaniasis and it was argued that the response to PM varied with the species and the type of lesion being treated³¹. These studies have also highlighted the need for a rational pharmaceutical design of formulations optimal for CL and the need for species specific diagnosis³².

Amphotericin B formulations: Amphotericin B (Amp B) in the form of amphotericin B deoxycholate (Fungizone®) is the second-line treatment for visceral leishmaniasis when antimonial therapy fails. Originally developed as a systemic antifungal, it is also an efficient antileishmanial, but has the major drawback of being acutely toxic and thus must be carefully administered. To ameliorate this, re-formulations of Amp B have been developed to alter its pharmacokinetics. By changing the serum-binding properties, its high affinity for low density lipoproteins (LDL) being the major cause of toxicity, lipid associated Amp B preparations have been made with varying degrees of success. The liposomal amphotericin B formulation, AmBisome®, is registered treatment for visceral leishmaniasis³³, but use in VL endemic regions is limited by cost. Other commercial amphotericin B - lipid formulations have also been manufactured, namely an amphotericin B lipid complex (Abelcet®) and an amphotericin B colloidal dispersion (Amphocil™)³⁴ but their use against VL has not been as extensive as AmBisome® and they too, are costly³⁵. Other re-formulations of amp B formulations have been investigated against experimental VL but none have reached clinical development to date. Approaches to reduce cost include: (i) efficacy trials of single dose AmBisome treatment for VL, with 90 per cent cure rate reported to date¹, and (ii) the use of cheaper liposomal formulations, already tried for VL³⁶. Alternative amphotericin B formulations have been developed. For example, arabinogalactan derivatives^{36,37}, nanoparticles^{38,39}; and other lipid formulations⁴⁰, or chemical derivatives⁴¹, have proved effective in

experimental models^{42,43}. The act of heating amphotericin B to form superaggregates reduces *in vivo* toxicity without loss of efficacy and warrants further investigation as a cheap alternative to the lipid formulations^{44,45}. It has already been evaluated for the treatment of canine VL⁴⁶.

Novel amphotericin B formulations have been used successfully to treat CL in immunocompromised patients⁴⁷ and paediatric CL⁴³. However, as CL is usually a self-limiting syndrome little effort has been made to evaluate the use of amphotericin B as a treatment for CL on a wider scale⁴⁸.

Sitamaquine: Another oral drug that might have an impact on VL is the 8-aminoquinoline derivative sitamaquine, currently in development with GlaxoSmithKline (GSK, <http://www.gsk.com>)⁴⁹. The antileishmanial activity of this compound was first identified in the 1970s at the Walter Reed Army Institute of Research (WRAIR, <http://wrair-www.army.mil/>). Limited Phase I/II clinical trials have been completed with varying levels of success, for instance, 67 per cent of patients were cured of *L. chagasi* in Brazil when treated with 2 mg/kg daily for 28 days⁵⁰, and 92 per cent were cured of VL when treated with 1.7 mg/kg daily for 28 days in Kenya⁵¹ and a 89 per cent cure rate with 1.75 mg/kg daily for 28 days in India⁵². Sitamaquine is rapidly metabolized, forming desethyl and 4-CH₂OH derivatives, which might be responsible for its activity. Toxicity appears to be relatively mild, it causes mild methemoglobinaemia, and further studies are underway on this drug.

Imiquimod: Imiquimod (Aldara, 3M Pharmaceuticals) is an antiviral compound [1-(2-methylpropyl)-1*H*-imidazo(4,5-*c*)quinolin-4-amine] used extensively for the topical treatment of genital warts caused by the human papillomavirus. It is an immunomodulator, stimulating a local immune response at the site of application, which in turn resolves the infection. Imiquimod induces the production of cytokines and nitric oxide in macrophages and has been shown to have an effect

in experimental infections of cutaneous leishmaniasis⁵³, and in conjunction with standard antimonial chemotherapy, has been used to successfully treat patients with cutaneous lesions which did not respond to antimonial therapy alone⁵⁴. It is suggested that the topical treatment activates localized macrophages to kill the parasite, while the antimonial eliminates systemic amastigotes which are responsible for persistence of infection^{55,56}.

Drugs in lead optimization and preclinical phases

Although many compounds have shown activity in *in vitro* models, few have received thorough testing in rodent models of infection. Of these, a few have demonstrated significant antileishmanial activity in different models. Plant products are an abundant source of leads, evidenced in the area of antimalarials. Licochalcone A from the Chinese liquorice plant *Glycyrrhiza* has shown reasonable oral efficacy in experimental models of VL and CL; synthetic oxygenated derivatives are also active⁵⁷. One derivative, 35m4ac, gave 97 per cent suppression of *L. donovani* liver amastigotes in a hamster model at 20 mg/kg for 6 days i.p. The compounds appear to interfere with mitochondrial function. Another compound, PX-6581, from the Vietnamese plant *Maesa balansae*, showed significant activity in VL models but was not progressed due to toxicity^{58,59}. Further study on this series to optimize activity is underway⁶⁰. Isopropylquinolines, isolated from *Galipea longiflora* in Bolivia, also showed activity in VL and CL models⁶¹ and studies on further SAR have recently been published⁶². Other quinoline derivatives, the indolyl quinolines, have also shown activity against VL in murine models and are the subject of further research⁶³.

Therapeutic switching or “piggy-back chemotherapy” is another potential source for new antileishmanial compounds. Azoles, originally developed as antifungal drugs, have shown activity against *Leishmania* spp. Like fungi, *Leishmania* synthesize 24-substituted sterols, such as ergosterol

(mammals have cholesterol). Azoles can inhibit a key enzyme of this pathway, 14 α -demethylase. Ketoconazole, itraconazole and fluconazole have given equivocal results in trials against both CL and VL⁶⁴. Oral posaconazole has shown encouraging activity against experimental *L. amazonensis*⁶⁵ but this has not been developed further. Bisphosphonates, used for the treatment of bone disorders such as osteoporosis, are another example of therapeutic switching. Two of these drugs, risedronate and pamidronate, were active against experimental infections of both CL and VL^{66,67}.

Drug discovery and development

The overall stages of drug discovery and development for neglected diseases, like leishmaniasis, have been recently described⁶⁸ and will not be covered here. We will focus here on the specific *in vitro* and *in vivo* assays required in the drug discovery process for leishmaniasis.

In vitro assays: In an earlier review⁶⁹, requirements for an *in vitro* assay to indicate the intrinsic activity of antileishmanial drugs were outlined and included use of (i) mammalian stage of the parasite, (ii) a dividing population, (iii) quantifiable and reproducible measures of drug activity, and (iv) activity of standard drugs in concentrations achievable in serum/tissues. Recently, assay design has focussed on features that make the assay adaptable to medium throughput screening (MTS), with additional requirements of (i) small amounts of compound (<1 mg), (ii) quick throughput, and (iii) low cost of tests. Other useful features of *in vitro* assays are adaptability for studies on (i) variation of drug sensitivity using recent isolates, different species/strains, resistant strains, and (ii) effects of immune or metabolic components.

For antileishmanial drug discovery *in vitro* assays are available on:

(i) *Promastigotes*: Drug activity against this extracellular stage is easy to determine. However,

there are significant differences between promastigotes and amastigotes in biochemistry and sensitivity to standard and experimental drugs^{13,70-72}. Promastigotes assays are useful cytotoxicity indicators in bioassay-guided fractionation of plant products.

(ii) *Macrophage - amastigote models*: The most widely used models for testing drugs against *Leishmania* species have involved either murine peritoneal macrophages or human-monocyte transformed macrophages as host cells. These models are able to show species/strain variation in drug sensitivity^{13,14}. In these differentiated non-dividing macrophages the rate of amastigote division in host cells and drug activity can be clearly determined. Drug activity is measured by either microscopical counting of percentage of infected cells or number of amastigotes/macrophage⁷³ or colorimetric or fluorometric methods. The slow rate of division of *L. donovani* and *L. infantum* amastigotes in this

model is a limitation. Mouse (J774)⁷⁴ and human (THP-1, U937, HL-60) monocytic cell lines have been used in drug assays⁷⁵. Assays that use dividing host cells must ensure that the confounding effects of drug activity on both parasite and host cell number are considered. THP-1 cells can form a non-dividing monolayer and make an attractive alternative to primary isolated macrophages, and have been used in antibacterial assays⁷⁶.

(iii) *Axenic amastigotes*: Axenic amastigote cultures offer different opportunities and protocols have been described for *L. mexicana*^{77,78}, *L. braziliensis*⁷⁹, *L. donovani*^{80,81} and *L. infantum*⁸². However, the amastigotes used must have confirmed biochemical and immunological markers and interpretation of data related to the high concentration of serum that is required in some systems. Differences in drug sensitivity between axenic *L. donovani* amastigotes and intracellular amastigotes have been observed⁸¹.

Table. Current drugs used for the treatment of leishmaniasis

Visceral leishmaniasis

First line drugs Sodium stibogluconate (Pentostam, SSG); meglumine antimoniate (Glucantime)
Amphotericin B (Fungizone)
Liposomal amphotericin B (AmBisome)
Pentamidine

Clinical trials Miltefosine (oral, Phase IV; registered in India)
Paromomycin (Phase III)
Sitamaquine (oral, Phase II)
Other amphotericin B formulations

Cutaneous leishmaniasis:

First line drugs Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime)
Amphotericin B (Fungizone)
Pentamidine
Paromomycin (topical formulations with methylbenzethonium chloride or urea)

Clinical trials Miltefosine (oral, Phase III, registered in Colombia)
Paromomycin (topical formulation with gentamicin and surfactants, Phase II)
Imiquimod (topical immunomodulator, Phase II)
Also anti-fungal azoles – ketoconazole, fluconazole, itraconazole

Parenteral administration unless otherwise stated

(iv) *Automated screening*: A major limitation of the amastigote-macrophage model is that the absence of automation and dependence on microscopical evaluation. Promastigote assays using reazzurin (Alamar Blue) and transfected parasites have been successful^{83,84} but not within the clinically relevant amastigote-macrophage model. Various groups have successfully transfected reporter genes into *Leishmania*, however, the majority of them require drug selection to maintain the plasmid, as in *Trypanosoma cruzi*⁸⁵. This is not ideal for use in drug experiments. Recently the firefly luciferase gene has been successfully integrated into the genome of a *L. amazonensis* strain and has been evaluated *in vitro*. It was used in a drug-screening assay in 96-well plates and compared favourably with microscopical evaluation⁸⁵. Transfected reference strains of *Leishmania*, such as *L. donovani* HU3, would be of further use to the drug screening community and work is underway to achieve this.

In vivo assays: Animal models enable drug activity to be determined in relation to absorption (route of administration), distribution (different sites of infection), metabolism (pro-drugs, immunomodulators), excretion and to give an early indication of the toxicity. Most of the models use mice, where a relatively low amount of compound is required, which are available as SPF and inbred strains enabling reproducible results with five animals/group. Mice are susceptible to most strains and species of *Leishmania* in both non-cure and self-cure models. The aim of using the animal model is to find a drug that can be administered orally, be effective in a short course (< 10 days) and have no indication of toxicity at the highest doses tested (100 mg/kg). (i) For visceral leishmaniasis inbred strains of mice are widely used with susceptible, resistant and intermediate strains being available. The BALB/c mouse is a commonly used strain, at 18-20 g, with highly reproducible levels of infection when an amastigote inoculum is administered i.v.. An assay in week two after infection examines the activity of the drug against the liver infection but

not the spleen infection. The infection in each mouse strain needs to be characterized for each parasite strain used to ensure that drugs are tested appropriately. Athymic and *scid* mice provide a model for treatment of VL in immunosuppressed cases^{87,88}. The hamster is also a good model for VL and provides a more synchronous infection in the liver and spleen that can develop into a chronic non-cure infection more similar to human VL. However, the weight (50-60 g), the absence of an easy route for i.v. inoculation and the limited availability of inbred SPF animals make this model less predictable. A problem in all the models is the determination of drug activity upon necropsy or biopsy which has been dependent on microscopy to determine the level of infection. This is now being replaced by quantitative PCR techniques⁹⁰. (ii) For cutaneous leishmaniasis inbred strains of mice are available with defined susceptibility/resistance, cure and non-cure to *L. major*, *L. mexicana* and *L. panamensis*. The BALB/c mouse - *L. major* model has been used widely in drug studies but it is an exceptionally rigorous non-cure model in which only the most active drugs have any efficacy and absolute cure is rare. In this model the standard antimonial drugs are ineffective. Other mouse models (CBA, C57/Bl) that self-cure, like most humans, should be used for studies on lead compounds⁹⁰. Lesions on the back of mice are also amenable for testing topical formulations. The determination of drug efficacy by measuring changes in lesion size (three dimensions) during and after the course of treatment can be misleading as much of the lesion is composed of inflammatory cells with amastigotes restricted to dermal layer of the skin. Culturing parasites from biopsies provides an alternative measurement of activity and has been shown to give quantitative data^{91,92}. However, PCR methodologies have been introduced that give quantitative and reproducible data^{93,94}. Poor animal models for *L. tropica*, *L. aethiopica* and *L. braziliensis* are a limitation for studies. Recent work on the *L. braziliensis* hamster model has shown potential for drug testing⁹⁵.

Concluding remarks

The drug discovery pipeline for leishmaniasis is imbalanced. With data from the genome project complied with interest from key researchers in biochemistry and chemistry, there has been renewed interest in drug discovery. Projects have identified many hits and leads. However, like in many areas of drug research, the tools, models and skills available to progress these compounds through the lead optimization and pre-clinical stages are limited. Along with efforts to find new compounds, resources availability is essential at this crucial stage of drug development to key groups and centres.

References

- Sundar S, Agrawal G, Rai M, Makharia MK, Murray HW. Treatment of Indian visceral leishmaniasis with single or daily infusions of low dose liposomal amphotericin B: randomised trial." *BMJ* 2001; 323 : 419-22.
- WHO. The Leishmaniases and *Leishmania*/HIV co-infections. Geneva: WHO/OMS; 1998.
- Dereure J, Thanh HD, Lavabre-Bertrand T, Cartron G, Bastides F, Richard-Lenoble D, *et al.* Visceral leishmaniasis. Persistence of parasites in lymph nodes after clinical cure. *J Infect* 2003; 47 : 77-81.
- Pagliano P, Rossi M, Rescigno C, Altieri S, Coppola MG, Gramiccia M, *et al.* Mediterranean visceral leishmaniasis in HIV-negative adults: a retrospective analysis of 64 consecutive cases (1995-2001). *J Antimicrob Chemother* 2003; 52 : 264-8.
- Mira JA, Corzo JE, Rivero A, Macias J, De Leon FL, Torre-Cisneros J, *et al.* Frequency of visceral leishmaniasis relapses in human immunodeficiency virus-infected patients receiving highly active antiretroviral therapy. *Am J Trop Med Hyg* 2004; 70 : 298-301.
- Croft SL, Coombs GH. Leishmaniasis- current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol* 2003; 19 : 502-8.
- Croft SL, Neal RA, Pendergast W, Chan JH. The activity of alkyl phosphorylcholines and related derivatives against *Leishmania donovani*. *Biochem Pharmacol* 1987; 36 : 2633-6.
- Kuhlencord A, Maniera T, Eibl H, Unger C. Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. *Antimicrob Agents Chemother* 1992; 36 : 1630-4.
- Croft SL, Snowdon D, Yardley V. The activities of four anticancer alkyllysophospholipids against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei*. *J Antimicrob Chemother* 1996; 38 : 1041-7.
- Bryceson A. A policy for leishmaniasis with respect to the prevention and control of drug resistance. *Trop Med Int Health* 2001; 6 : 928-34.
- Seifert K, Matu S, Javier Perez-Victoria F, Castanys S, Gamarro F, Croft SL. Characterisation of *Leishmania donovani* promastigotes resistant to hexadecylphosphocholine (miltefosine). *Int J Antimicrob Agents* 2003; 22 : 380-7.
- Perez-Victoria FJ, Gamarro F, Ouellette M, Castanys S. Functional cloning of the miltefosine transporter. A novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. *J Biol Chem* 2003; 278 : 49965-71.
- Escobar P, Matu S, Marques C, Croft SL. Sensitivities of *Leishmania* species to hexadecylphosphocholine (miltefosine), ET-18-OCH₃ (edelfosine) and amphotericin B. *Acta Trop* 2002; 81 : 151-7.
- Yardley V, Croft SL, De Doncker S, Dujardin JC, Koirala S, Rijal S, *et al.* The sensitivity of clinical isolates of *Leishmania* from Peru and Nepal to miltefosine. *Am J Trop Med Hyg* 2005; 73 : 272-5.
- Soto J, Arana BA, Toledo J, Rizzo N, Vega JC, Diaz A, *et al.* Miltefosine for new world cutaneous leishmaniasis. *Clin Infect Dis* 2004; 38 : 1266-72.
- Avlonitis N, Lekka E, Detsi A, Koufaki M, Calogeropoulou T, Scoulica E, *et al.* Antileishmanial ring-substituted ether phospholipids. *J Med Chem* 2003; 46 : 755-67.
- Lux H, Hart DT, Parker PJ, Klenner T. Ether lipid metabolism, GPI anchor biosynthesis, and signal transduction are putative targets for anti-leishmanial alkyl phospholipid analogues. *Adv Exp Med Biol* 1996; 416 : 201-11.
- Paris C, Loiseau PM, Bories C, Breard J. Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* 2004; 48 : 852-9.

19. Verma NK, Dey CS. Possible mechanism of miltefosine-related death of *Leishmania donovani*. *Antimicrob Agents Chemother* 2004; 48 : 3010-5.
20. Lux H, Heise N, Klenner T, Hart D, Opperdoes FR. Ether-lipid (alkyl-phospholipid) metabolism and the mechanism of action of ether - lipid analogues in *Leishmania*. *Mol Biochem Parasitol* 2000; 111 : 1-14.
21. Seifert K, Croft SL. *In vitro* and *in vivo* interactions between miltefosine and other antileishmanial drugs. *Antimicrob Agents Chemother* 2006; 50 : 73-9.
22. Maarouf M, Adeline MT, Solignac M, Vautrin D, Robert-Gero M. Development and characterization of paromomycin-resistant *Leishmania donovani* promastigotes. *Parasite* 1998; 5 : 167-73.
23. Maarouf M, Lawrence F, Brown S, Robert-Gero M. Biochemical alterations in paromomycin-treated *Leishmania donovani* promastigotes. *Parasitol Res* 1997; 83 : 198-202.
24. Maarouf M, Lawrence F, Croft SL, Robert-Gero M. Ribosomes of *Leishmania* are a target for the aminoglycosides. *Parasitol Res* 1995; 81 : 421-5.
25. Neal RA, Allen S, McCoy N, Olliaro P, Croft SL. The sensitivity of *Leishmania* species to aminosidine. *J Antimicrob Chemother* 1995; 35 : 577-84.
26. Seaman J, Boer C, Wilkinson R, de Jong J, de Wilde E, Sondorp E. Liposomal amphotericin B (AmBisome) in the treatment of complicated kala-azar under field conditions." *Clin Infect Dis* 1995; 21 : 188-93.
27. Thakur CP, Kanyok TP, Pandey AK, Sinha GP, Zaniewski AE, Houlihan HH, *et al.* A prospective, randomised, comparative, open-label trial of the safety and efficacy of paromomycin (aminosidine) plus sodium stibogluconate versus sodium stibogluconate alone for the treatment of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 2000; 94 : 429-31.
28. El-On J, Jacobs GP, Witztum E, Greenblatt C. Development of topical treatment for cutaneous leishmaniasis caused by *Leishmania major* in experimental animals. *Antimicrob Agents Chemother* 1984; 26 : 745-51.
29. El-On J, Halevy S, Grunwald MH, Weinrauch L. Topical treatment of Old World cutaneous leishmaniasis caused by *Leishmania major*: a double-blind control study. *J Am Acad Dermatol* 1992; 27 : 227-31.
30. Soto JM, Toledo JT, Gutierrez P, Arboleda M, Nicholls RS, Padilla JR, *et al.* Treatment of cutaneous leishmaniasis with a topical antileishmanial drug (WR279396): phase 2 pilot study. *Am J Trop Med Hyg* 2002; 66 : 147-51.
31. Iraj F, Sadeghinia A. Efficacy of paromomycin ointment in the treatment of cutaneous leishmaniasis: results of a double-blind, randomized trial in Isfahan, Iran. *Ann Trop Med Parasitol* 2005; 99 : 3-9.
32. Garnier TS, Croft SL. Topical treatment for cutaneous leishmaniasis. *Curr Opin Investig Drugs* 2002; 3 : 538-44.
33. Meyerhoff A. U.S. Food and Drug Administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. *Clin Infect Dis* 1999; 28 : 42-8; discussion 49-51.
34. Robinson RF, Nahata MC. A comparative review of conventional and lipid formulations of amphotericin B. *J Clin Pharm Ther* 1999; 24 : 249-57.
35. Murray HW. Progress in the treatment of a neglected infectious disease: visceral leishmaniasis. *Expert Rev Anti Infect Ther* 2004; 2 : 279-92.
36. Falk R, Domb AJ, Polachek I. A novel injectable water-soluble amphotericin B-arabinogalactan conjugate. *Antimicrob Agents Chemother* 1999; 43 : 1975-81.
37. Golenser J, Frankenburg S, Ehrenfreund T, Domb AJ. Efficacious treatment of experimental leishmaniasis with amphotericin B-arabinogalactan water-soluble derivatives. *Antimicrob Agents Chemother* 1999; 43 : 2209-14.
38. Espuelas MS, L P, *et al.* Poly(epsilon-caprolacton) nanospheres as an alternative way to reduce amphotericin B toxicity. *Int J Pharmaceutics* 1997; 158 : 19-27.
39. Muller RH, Mader K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of the state of the art. *Eur J Pharm Biopharm* 2000; 50 : 161-77.
40. Larabi M, Yardley V, Loiseau PM, Appel M, Legrand P, Gulik A, *et al.* Toxicity and antileishmanial activity of a new stable lipid suspension of amphotericin B. *Antimicrob Agents Chemother* 2003; 47 : 3774-9.
41. Al-Abdely HM, Graybill JR, Bocanegra R, Najvar L, Montalbo E, Regan SL, *et al.* Efficacies of KY62 against *Leishmania amazonensis* and *Leishmania donovani* in

- experimental murine cutaneous leishmaniasis and visceral leishmaniasis. *Antimicrob Agents Chemother* 1998; 42 : 2542-8.
42. Petit C, Yardley V, Gaboriau F, Bolard J, Croft SL. Activity of a heat-induced reformulation of amphotericin B deoxycholate (fungizone) against *Leishmania donovani*. *Antimicrob Agents Chemother* 1999; 43 : 390-2.
43. Zvulunov A, Cagnano E, Frankenburg S, Barenholz Y, Vardy D. Topical treatment of persistent cutaneous leishmaniasis with ethanolic lipid amphotericin B." *Pediatr Infect Dis J* 2003; 22 : 567-9.
44. Hartsel SC, Baas B, Bauer E, Foree Jr LT, Kindt K, Preis H, et al. Heat-induced superaggregation of amphotericin B modifies its interaction with serum proteins and lipoproteins and stimulation of TNF-alpha. *J Pharm Sci* 2001; 90 : 124-33.
45. Kwong EH, Ramaswamy M, Bauer EA, Hartsel SC, Wasan KM. Heat treatment of amphotericin B modifies its serum pharmacokinetics, tissue distribution, and renal toxicity following administration of a single intravenous dose to rabbits. *Antimicrob Agents Chemother* 2001; 45 : 2060-3.
46. Lamothe J. A new formulation of Fungizone in the canine model of visceral leishmaniasis: amphotericin B treated by heat. WorldLeish3, Sicily, Italy; 2005.
47. Amato VS, Rabello A, Rotondo-Silva A, Kono A, Maldonado TP, Alves IC, et al. Successful treatment of cutaneous leishmaniasis with lipid formulations of amphotericin B in two immunocompromised patients. *Acta Trop* 2004; 92 : 127-32.
48. Yardley V, Croft SL. A comparison of the activities of three amphotericin B lipid formulations against experimental visceral and cutaneous leishmaniasis. *Int J Antimicrob Agents* 2000; 13 : 243-8.
49. Yeates C. Sitamaquine (GlaxoSmithKline/Walter Reed Army Institute). *Curr Opin Investig Drugs* 2002; 3 : 1446-52.
50. Dietze R, Carvalho SF, Valli LC, Berman J, Brewer T, Milhous W, et al. Phase 2 trial of WR6026, an orally administered 8-aminoquinoline, in the treatment of visceral leishmaniasis caused by *Leishmania chagasi*. *Am J Trop Med Hyg* 2001; 65 : 685-9.
51. Wasunna MK, Rashid JR, Mbui J, Kirigi G, Kinoti D, Lodenyo H, et al. A phase II dose-increasing study of sitamaquine for the treatment of visceral leishmaniasis in Kenya. *Am J Trop Med Hyg* 2005; 73 : 871-6.
52. Jha TK, Sundar S, Thakur CP, Felton MJ, Sabin AJ, Horton J. A phase II dose-ranging study of sitamaquine for the treatment of visceral leishmaniasis in India. *Am J Trop Med Hyg* 2005; 73 : 1005-11.
53. Buates S, Matlashewski G. Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: efficacy and mode of action. *J Infect Dis* 1999; 179 : 1485-94.
54. Arevalo I, Ward B, Miller R, Meng TC, Najjar E, Alvarez E, et al. Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of Imiquimod, an immunomodulator. *Clin Infect Dis* 2001; 33 : 1847-51.
55. Bogdan C, Donhauser N, Doring R, Rollinghoff M, Diefenbach A, Ritting MG. Fibroblasts as host cells in latent leishmaniasis. *J Exp Med* 2000; 191 : 2121-30.
56. Nicolas L, Sidjanski S, Colle JH, Milon G. *Leishmania major* reaches distant cutaneous sites where it persists transiently while persisting durably in the primary dermal site and its draining lymph node: a study with laboratory mice. *Infect Immun* 2000; 68 : 6561-6.
57. Zhai L, Chen M, Blom J, Theander TG, Christensen SB, Kharazmi A. The antileishmanial activity of novel oxygenated chalcones and their mechanism of action. *J Antimicrob Chemother* 1999; 43 : 793-803.
58. Maes L, Berghe DV, Germonprez N, Quirijnen L, Cos P, De Kimpe N, et al. *In vitro* and *in vivo* activities of a triterpenoid saponin extract (PX-6518) from the plant *Maesa balansae* against visceral *Leishmania* species. *Antimicrob Agents Chemother* 2004; 48 : 130-6.
59. Maes L, Germonprez N, Quirijnen L, Van Puyvelde L, Cos P, Vanden Berghe D. Comparative activities of the triterpene saponin Maesabalide III and liposomal amphotericin B (AmBisome) against *Leishmania donovani* in hamsters. *Antimicrob Agents Chemother* 2004; 48 : 2056-60.
60. Germonprez N, Maes L, Van Puyvelde L, Van Tri M, Tuan DA, De Kimpe N. *In vitro* and *in vivo* anti-leishmanial activity of triterpenoid saponins isolated from *Maesa balansae* and some chemical derivatives. *J Med Chem* 2005; 48 : 32-7.
61. Fournet A, Ferreira ME, Rojas De Arias A, Torres De Ortiz A, Fuentes S, Nakayama H, et al. *In vivo* efficacy of oral and intralésional administration of 2- substituted quinolines in experimental treatment of new world cutaneous leishmaniasis caused by *Leishmania amazonensis*. *Antimicrob Agents Chemother* 1996; 40 : 2447-51.

62. Franck X, Fournet A, Prina E, Mahieux R, Hocquemiller R, Figadere B. Biological evaluation of substituted quinolines. *Bioorg Med Chem Lett* 2004; 14 : 3635-8.
63. Chakrabarti G, Basu A, Manna PP, Mahato SB, Mandal NB, Bandyopadhyay S. Indolylquinoline derivatives are cytotoxic to *Leishmania donovani* promastigotes and amastigotes *in vitro* and are effective in treating murine visceral leishmaniasis. *J Antimicrob Chemother* 1999; 43 : 359-66.
64. Navin TR, Arana BA, Arana FE, Berman JD, Chajon JF. Placebo-controlled clinical trial of sodium stibogluconate (Pentostam) versus ketoconazole for treating cutaneous leishmaniasis in Guatemala. *J Infect Dis* 1992; 165 : 528-34.
65. Al-Abdely HM, Graybill JR, Loebenberg D, Melby PC. Efficacy of the triazole SCH 56592 against *Leishmania amazonensis* and *Leishmania donovani* in experimental murine cutaneous and visceral leishmaniases. *Antimicrob Agents Chemother* 1999; 43 : 2910-4.
66. Rodriguez N, Bailey BN, Martin MB, Oldfield E, Urbina JA, Docampo R. Radical cure of experimental cutaneous leishmaniasis by the bisphosphonate pamidronate. *J Infect Dis* 2002; 186 : 138-40.
67. Yardley V, Khan AA, Martin MB, Slifer TR, Araujo FG, Moreno SN, *et al.* *In vivo* activities of farnesyl pyrophosphate synthase inhibitors against *Leishmania donovani* and *Toxoplasma gondii*. *Antimicrob Agents Chemother* 2002; 46 : 929-31.
68. Nwaka S, Ridley RG. Virtual drug discovery and development for neglected diseases through public-private partnerships. *Nat Rev Drug Discov* 2003; 2 : 919-28.
69. Croft SL. *In vitro* screens in the experimental chemotherapy of leishmaniasis and trypanosomiasis. *Parasitol Today* 1986; 2 : 64-9.
70. Neal RA. *Leishmania major*: culture media, mouse strains, and promastigote virulence and infectivity. *Exp Parasitol* 1984; 57 : 269-73.
71. Carrio J, de Colmenares M, Riera C, Gallego M, Arboix M, Portus M. *Leishmania infantum*: stage-specific activity of pentavalent antimony related with the assay conditions. *Exp Parasitol* 2000; 95 : 209-14.
72. Agnew P, Holzmuller P, Michalakis Y, Sereno D, Lemesre JL, Renaud F. *In vitro* growth of *Leishmania amazonensis* promastigotes resistant to pentamidine is dependent on interactions among strains. *Antimicrob Agents Chemother* 2001; 45 : 1928-9.
73. Neal RA, Croft SL. An *in vitro* system for determining the activity of compounds against the intracellular amastigote form of *Leishmania donovani*. *J Antimicrob Chemother* 1984; 14 : 463-75.
74. Tempone AG, Perez D, Rath S, Vilarinho AL, Mortara RA, de Andrade HF. Targeting *Leishmania (L.) chagasi* amastigotes through macrophage scavenger receptors: the use of drugs entrapped in liposomes containing phosphatidylserine. *J Antimicrob Chemother* 2004; 54 : 60-8.
75. Gebre-Hiwot A, Tadesse G, Croft SL, Frommel D. An *in vitro* model for screening antileishmanial drugs: the human leukaemia monocyte cell line, THP-1. *Acta Trop* 1992; 51 : 237-45.
76. Carryn S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM. Activity of beta-lactams (ampicillin, meropenem), gentamicin, azithromycin and moxifloxacin against intracellular *Listeria monocytogenes* in a 24 h THP-1 human macrophage model. *J Antimicrob Chemother* 2003; 51 : 1051-2.
77. Bates PA. Complete developmental cycle of *Leishmania mexicana* in axenic culture. *Parasitology* 1994; 108 : 1-9.
78. Callahan HL, Portal AC, Devereaux R, Groggl M. An axenic amastigote system for drug screening. *Antimicrob Agents Chemother* 1997; 41 : 818-22.
79. Balanco JM, Pral EM, da Silva S, Bijovsky AT, Mortara RA, Alfieri SC. Axenic cultivation and partial characterization of *Leishmania braziliensis* amastigote-like stages. *Parasitology* 1998; 116 : 103-13.
80. Sereno D, Lemesre JL. Axenically cultured amastigote forms as an *in vitro* model for investigation of antileishmanial agents. *Antimicrob Agents Chemother* 1997; 41 : 972-6.
81. Ephros M, Bitnun A, Shaked P, Waldman E, Zilberstein D. Stage-specific activity of pentavalent antimony against *Leishmania donovani* axenic amastigotes. *Antimicrob Agents Chemother* 1999; 43 : 278-82.
82. Sereno D, Holzmuller P, Lemesre JL. Efficacy of second line drugs on antimony-resistant amastigotes of *Leishmania infantum*. *Acta Trop* 2000; 74 : 25-31.
83. Okuno T, Goto Y, Matsumoto Y, Otsuka H, Matsumoto Y. Applications of recombinant *Leishmania amazonensis* expressing *egfp* or the β -galactosidase gene for drug

- screening and histopathological analysis. *Exp Anim* 2003; 52 : 109-18.
84. Singh N, Dube A. Short report: fluorescent *Leishmania*: application to anti-leishmanial drug testing. *Am J Trop Med Hyg* 2004; 71 : 400-2.
85. Buckner FS, Verlinde CL, La Flamme AC, Van Voorhis WC. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing β -galactosidase. *Antimicrob Agents Chemother* 1996; 40 : 2592-7.
86. Lang T, Goyard S, Lebastard M, Milon G. Bioluminescent *Leishmania* expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harboured macrophages and for quantitative real-time monitoring of parasitism features in living mice. *Cell Microbiol* 2005; 7 : 383-92.
87. Marshall BG, Kropf P, Murray K, Clark C, Flanagan AM, Favidson RN, *et al.* Bronchopulmonary and mediastinal leishmaniasis: An unusual clinical presentation of *Leishmania donovani* infection. *Clin Infect Dis* 2000; 30 : 764-9.
88. Escobar P, Yardley V, Croft SL. Activities of Hexadecylphosphocholine (Miltefosine), AmBisome, and Sodium Stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficient scid Mice. *Antimicrob Agents Chemother* 2001; 45 : 1872-5.
89. Singh S, Dey A, Sivakumar R. Applications of molecular methods for *Leishmania* control. *Expert Rev Mol Diagn* 2005; 5 : 251-65.
90. Yardley V, Croft SL. Animal models of cutaneous leishmaniasis. In: *Handbook of animal models of infection*. Zak O, editor. New York London: Academic Press; 1999 p. 775-81.
91. Samuelson J, Lerner E, Tesh R, Titus R. A mouse model of *Leishmania braziliensis braziliensis* infection produced by coinjection with sand fly saliva. *J Exp Med* 1991; 173 : 49-54.
92. Andrade SG, Carneiro Filho A, de Souza AJ, de Lima ES, Andrade ZA. Influence of treatment with immunosuppressive drugs in mice chronically infected with *Trypanosoma cruzi*. *Int J Exp Pathol* 1997; 78 : 391-9.
93. Bretagne S, Durand R, Olivi M, Garin JF, Sulahian A, Rivollet D, *et al.* Real-time PCR as a new tool for quantifying *Leishmania infantum* in liver in infected mice. *Clin Diagn Lab Immunol* 2001; 8 : 828-31.
94. Nicolas L, Prina E, Lang T, Milon G. Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues. *J Clin Microbiol* 2002; 40 : 1666-9.
95. Goncalves GS, Fernandes AP, Souza RC, Cardoso JE, de Oliviera-Silva F, Maciel FC, *et al.* Activity of a paromomycin hydrophilic formulation for topical treatment of infections by *Leishmania (Leishmania) amazonensis* and *Leishmania (Vianna) braziliensis*. *Acta Trop* 2005; 93 : 161-7.

Reprint requests: Dr Simon Croft, DNDi, 1 Place St. Gervais, CH-1201 Geneva, Switzerland
e-mail: scroft@dndi.org