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Current scenario of drug development for leishmaniasis

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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, alklyphosphocholines 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
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 unsuccessful\(^2\)-\(^5\).

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 market\(^6\). 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 resistance\(^10\). It has been shown in vitro in laboratory studies on promastigotes that miltefosine resistant lines of *Leishmania donovani* can be selected\(^11\) and resistance is related to two mutations on a transporter, the aminophospholipid translocase LdMT\(^12\). Variation in species sensitivity, demonstrated in vitro with *L. donovani*, *L. aethiopica*, *L. tropica*, *L. mexicana* and *L. panamensis* being more sensitive (EC\(_{50}\) values between 2.63 and 10.63 µM) to miltefosine than *L. major* (EC\(_{50}\) value 37.17 µM)\(^13\), is also a concern. The variation, also demonstrated in clinical isolates\(^14\), 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 Guatemala\(^15\). 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 \textit{L.v. braziliensis} and \textit{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 \textit{L. donovani} and \textit{L. infantum} promastigotes and cytotoxicity towards THP-1 cells\textsuperscript{16}. With regard to headgroup SAR, choline is preferred resulting in similar activity against both \textit{Leishmania} strains, whereas the introduction of cycloalkylidene groups in the lipid portion results in enhanced activity, more pronounced against \textit{L. infantum} than \textit{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 apoptosis\textsuperscript{17-19}, as well as inhibition of an alkyl-specific acyl-CoA acyltransferase in \textit{L. mexicana}\textsuperscript{20}. Miltefosine also displayed marked activity against another trypanosomatid, \textit{Trypanosoma cruzi}, with EC\textsubscript{50} values < 2 µM against intracellular amastigotes of the Y strain in murine macrophages\textsuperscript{9}. Further studies on miltefosine of basic and preclinical nature are underway, including interaction studies for the potential use of drug combinations\textsuperscript{21}.

\textbf{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 \textit{L. donovani} promastigotes experimentally \textit{in vitro}. The resistance was specific to PM and stable and its mechanism seems to be due to decreased drug uptake\textsuperscript{22}. Ribosomes have been implicated as target\textsuperscript{23} and inhibition of RNA synthesis followed by protein synthesis shown, along with induction of respiratory dysfunction\textsuperscript{24}. With PM moving into the field more studies on the mechanism of action and, more importantly, resistance are required. Variation in species sensitivity \textit{in vitro} has also been demonstrated for PM with \textit{L. braziliensis} and \textit{L. mexicana} being less sensitive (EC\textsubscript{50} values between 38 and 39.4 µM) than \textit{L. major}, \textit{L. tropica} and \textit{L. panamensis} (EC\textsubscript{50} values between 0.9 and 4.9 µM)\textsuperscript{25}. It is interesting to note the considerable differences between different isolates of \textit{L. donovani} (EC\textsubscript{50} 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 \textit{L. donovani} marked synergy with sodium stibogluconate was demonstrated \textit{in vitro}, to a lesser extent against an Indian strain (MHOM/IN/82/PATNA I). The interaction \textit{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\textsuperscript{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 1984\textsuperscript{28} 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 group\textsuperscript{29}. 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 Colombia. 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 treated. These studies have also highlighted the need for a rational pharmaceutical design of formulations optimal for CL and the need for species specific diagnosis.

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, reformulations 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 leishmaniasis, 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™) but their use against VL has not been as extensive as AmBisome® and they too, are costly. 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 date, and (ii) the use of cheaper liposomal formulations, already tried for VL. Alternative amphotericin B formulations have been developed. For example, arabinogalactan derivatives, nanoparticles, and other lipid formulations, or chemical derivatives, have proved effective in experimental models. 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. It has already been evaluated for the treatment of canine VL.

Novel amphotericin B formulations have been used successfully to treat CL in immunocompromised patients and paediatric CL. 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 scale.

Sitamaquine: Another oral drug that might have an impact on VL is the 8-aminooquinoline derivative sitamaquine, currently in development with GlaxoSmithKline (GSK, http://www.gsk.com). 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 days, and 92 per cent were cured of VL when treated with 1.7 mg/kg daily for 28 days in Kenya and a 89 per cent cure rate with 1.75 mg/kg daily for 28 days in India. Sitamaquine is rapidly metabolized, forming desethyl and 4-CH 2 OH derivatives, which might be responsible for its activity. Toxicity appears to be relatively mild, it causes mild methemoglobinemia, and further studies are underway on this drug.

Imiquimod: Imiquimod (Aldara, 3M Pharmaceuticals) is an antiviral compound [1-(2-methylpropyl)-1H-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 on the immune system.
in experimental infections of cutaneous leishmaniasis, and in conjunction with standard antimonial chemotherapy, has been used to successfully treat patients with cutaneous lesions which did not respond to antimonial therapy alone. 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.

**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 active. 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. Further study on this series to optimize activity is underway. Isopropylquinolines, isolated from *Galipea longiflora* in Bolivia, also showed activity in VL and CL models and are the subject of further research.

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, 14α-demethylase. Ketoconazole,itraconazole and fluconazole have given equivocal results in trials against both CL and VL. Oral posaconazole has shown encouraging activity against experimental *L. amazonensis* 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.

**Drug discovery and development**

The overall stages of drug discovery and development for neglected diseases, like leishmaniasis, have been recently described 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 review, 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. 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-monooye tranformed macrophages as host cells. These models are able to show species/strain variation in drug sensitivity. 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/macrophage 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) and human (THP-1, U937, HL-60) monocytic cell lines have been used in drug assays. 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 assays.

(iii) Axenic amastigotes: Axenic amastigote cultures offer different opportunities and protocols have been described for L. mexicana, L. braziliensis, L. donovani and L. infantum. 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 observed.

Table. Current drugs used for the treatment of leishmaniasis

<table>
<thead>
<tr>
<th>Visceral leishmaniasis</th>
<th>First line drugs</th>
<th>Sodium stibogluconate (Pentostam, SSG); meglumine antimoniate (Glucantime)</th>
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<tbody>
<tr>
<td></td>
<td>Amphotericin B (Fungizone)</td>
<td>Liposomal amphotericin B (AmBisome)</td>
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<td></td>
<td>Pentamidine</td>
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<tr>
<td>Clinical trials</td>
<td>Miltefosine (oral, Phase IV; registered in India )</td>
<td>Paromomycin (Phase III)</td>
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<td></td>
<td>Sitamamide (oral, Phase II)</td>
<td>Other amphotericin B formulations</td>
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<tr>
<td>Cutaneous leishmaniasis:</td>
<td>First line drugs</td>
<td>Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime)</td>
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<tr>
<td></td>
<td>Amphotericin B (Fungizone)</td>
<td>Pentamidine</td>
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<td></td>
<td>Paromomycin (topical formulations with methylbenzethonium chloride or urea)</td>
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</tr>
<tr>
<td>Clinical trials</td>
<td>Miltefosine (oral, Phase III, registered in Colombia)</td>
<td>Paromomycin (topical formulation with gentamicin and surfactants, Phase II)</td>
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<tr>
<td></td>
<td>Imiquimod (topical immunomodulator, Phase II)</td>
<td>Also anti-fungal azoles – ketoconazole, fluconazole, itraconazole</td>
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<tr>
<td>Parenteral administration unless otherwise stated</td>
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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 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. 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 evaluation. 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 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). (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 compounds. 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. However, PCR methodologies have been introduced that give quantitative and reproducible data. 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 testing.
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.

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