

Chemotherapy of leishmaniasis: present challenges

SILVIA R. B. ULIANA*, CRISTIANA T. TRINCONI and ADRIANO C. COELHO†

Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1374, 05508-000 São Paulo, Brazil

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SUMMARY

Cutaneous and visceral leishmaniasis are amongst the most devastating infectious diseases of our time, affecting millions of people worldwide. The treatment of these serious diseases rely on a few chemotherapeutic agents, most of which are of parenteral use and induce severe side-effects. Furthermore, rates of treatment failure are high and have been linked to drug resistance in some areas. Here, we reviewed data on current chemotherapy practice in leishmaniasis. Drug resistance and mechanisms of resistance are described as well as the prospects for applying drug combinations for leishmaniasis chemotherapy. It is clear that efforts for discovering new drugs applicable to leishmaniasis chemotherapy are essential. The main aspects on the various steps of drug discovery in the field are discussed.

Key words: leishmaniasis treatment, drug screening, animal models, combination therapy, drug resistance.

INTRODUCTION

The leishmaniasis, including the cutaneous and visceral forms (CL and VL) of the disease, are amongst the most devastating infectious diseases of our time. The most recent estimates report about 0.2–0.4 million VL cases and 0.7–1.2 million CL cases each year (Alvar *et al.* 2012). Leishmaniasis management includes multiple difficulties, such as vector and animal reservoir control, but probably the most challenging of them all is treatment.

Over 20 species within the genus are implicated as the aetiological agents of the human leishmaniasis in a spectrum of clinical manifestations. The visceral presentation is the most severe form and leads to death almost invariably if untreated. The aetiological agents for VL are *Leishmania donovani* and *Leishmania infantum* (in the Old World) and *L. infantum chagasi* (in the New World). The cutaneous or tegumentary form can be categorized as localized, diffuse, disseminated and mucocutaneous. Apart from the localized form, which can heal spontaneously after a chronic progression, all the other forms are severe, mutilating and respond poorly to the available therapeutic options. The most common agents of CL in the Old World are *Leishmania major* and *Leishmania tropica*, but *L. infantum* and *Leishmania aethiopica* are also detected in cutaneous patients. In the Americas, several species of the subgenus *Viannia* are responsible for human disease, the most common of them being *Leishmania*

braziliensis. In a more restricted geographical pattern, *Leishmania guyanensis*, *Leishmania panamensis* and *Leishmania peruviana* are also common, while *Leishmania shawi*, *Leishmania naiffi*, *Leishmania lainsoni* and *Leishmania lindenbergi* are diagnosed less often in the Amazon region. In the Americas, species of the *Leishmania* subgenus, such as *L. amazonensis*, *L. mexicana* and *L. venezuelensis* are also agents of CL. Post kalazar dermal leishmaniasis, a cutaneous presentation of *L. donovani* infection, often arises after treatment of the visceral disease, more frequently in East Africa and India.

According to the Global Burden of Disease Study 2013, from 2005 to 2013, the number of disability adjusted life years (DALYs) as a result of leishmaniasis increased 15% globally (Murray *et al.* 2016). When the clinical forms were considered, the rise in DALYs was 14.4% for VL and 175.1% for CL. In 33 out of 50 countries with the highest CL incidence, a rise in age-adjusted DALYs was observed between 1990 and 2013. The greatest increases were found in Sudan, Iraq, Gambia, Benin, Venezuela, Paraguay, Ecuador and Honduras (Karimkhani *et al.* 2016).

The factors behind this increase in incidence derive from climate change, dislocation of populations from endemic rural areas to new agricultural or urban zones (Gadisa *et al.* 2015), as well as the installation of conflict zones (Berry and Berrang-Ford, 2016).

Large epidemics of VL and CL have been registered in conflict areas such as Sudan, Iraq, Afghanistan and Syria (Kreutzer *et al.* 1993; Hyams *et al.* 2001; Collin *et al.* 2004; Berry and Berrang-Ford, 2016) not only resulting in large mortality rates, but also in the emergence of new foci of transmission (Alawieh *et al.* 2014; Sharara and Kanj,

* Corresponding author: Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1374, 05508-000 São Paulo, Brazil. E-mail: srbulian@icb.usp.br

† Present address: Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil.

2014; Inci *et al.* 2015), sometimes with unexpected clinical presentations (Kreutzer *et al.* 1993).

Geographical expansion outside of conflict zones has been registered as well. Factors most likely associated with these expansions are human migration and climate change.

LEISHMANIASIS TREATMENT

There are only a few drugs available for leishmaniasis treatment and most of them have been in use for quite some time (Table 1). Curiously, the approaches to leishmaniasis treatment are mostly very broad and not species-specific, in spite of all the clinical diversity and of the great deal of correlation between species-specific determinants of clinical disease patterns. The specific choice of drug or length of treatment generally does not take into account the particularities of the different aetiological agents, because there are no easily accessible methods for species-specific diagnosis, while the co-existence of different species in the same geographical area is common. Therefore, in general, researchers and clinicians have not been able to ascertain whether species-specific schemes of treatment are more appropriate than an overall strategy.

Pentavalent antimonials were the first class of drugs applied to leishmaniasis treatment. Together with amphotericin B, pentamidine, miltefosine and paromomycin, they constitute the resources available for leishmaniasis chemotherapy.

The initial observations that led to the global use of antimonials and amphotericin B as the mainstay of leishmaniasis therapy were made by Brazilians. Gaspar Vianna suggested trivalent antimonials could be useful to treat the first cases of CL diagnosed in São Paulo, in 1914 (Vianna, 1914). Pentavalent antimonials were developed during the 1920s, in India, and contributed to decrease considerably the treatment toxicity (Marsden, 1985). In the late 1950s, amphotericin B was described by Furtado and Lacaz as an alternative to the management of mucocutaneous leishmaniasis patients who frequently relapsed or did not respond to antimonial drugs (Sampaio *et al.* 1960) and was soon applied to the treatment of severe VL (Prata, 1963).

For decades, treatment schemes with pentavalent antimonials were used as the first choice to treat VL all over the world, in spite of the parenteral route of administration, high toxicity and cost. However, an increase in the number of therapeutic failures was noted from the 1980s in India and doses required to lead to clinical cure had to be slowly increased (Thakur, 1984; Thakur *et al.* 1984). WHO recommendations of pentavalent antimonial doses of $10 \text{ mg Sb}^{\text{V}} \text{ kg}^{-1} \text{ day}^{-1}$ for 20 days in 1984 (WHO, 1984) were raised to $20 \text{ mg kg}^{-1} \text{ day}^{-1}$ and the period of administration to 28–30 days. The latest WHO recommendations have


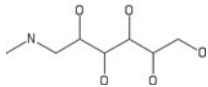
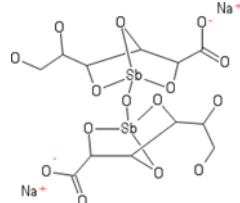
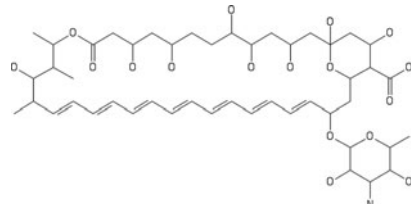
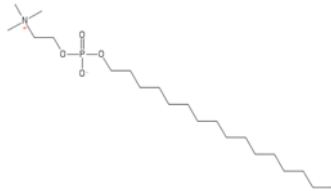
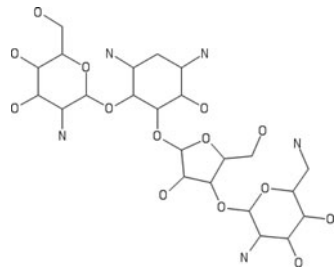
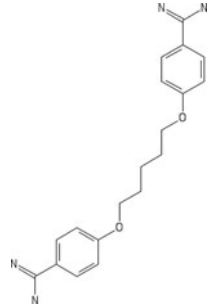
demoted the use of pentavalent antimonials as single drugs to second or lower choices for the treatment of VL in all endemic regions, making liposomal amphotericin B the first recommended drug in most areas (WHO, 2010). However, due to the constraints on liposomal amphotericin use, mostly related to price and availability, pentavalent antimonials are still largely used as the first option for VL in some endemic countries.

Amphotericin B deoxycholate and liposomal amphotericin B have been in use for the treatment of VL in the presence of concomitant illnesses or risk factors such as age extremes. Amphotericin B deoxycholate is highly toxic and has to be administered by slow intravenous infusion over 4 to 6 h, requiring hospitalization. Lipid formulations of amphotericin B display similar efficacy with significantly lower toxicity (Szoka *et al.* 1987; Botero Aguirre and Restrepo Hamid, 2015). These formulations are now recommended as the first choice drug, with a good level of evidence for efficacy (Balasegaram *et al.* 2012). However, its cost is still prohibitive in several endemic regions.

Another drug available for VL treatment is miltefosine (hexadecylphosphocholine), a phosphatidylcholine analogue, initially developed as an antineoplastic drug, that showed good activity against *L. donovani* (Croft *et al.* 1987; Kuhlencord *et al.* 1992) and was shown to be very effective for the treatment of VL in India, country that was the first to approve its clinical use in 2002 (Sundar *et al.* 2002). The main drawbacks of miltefosine are its teratogenic potential and the long half-life that can lead to the selection of drug-resistant lines (Dorlo *et al.* 2008). However, it benefits from being administered orally. Side-effects are mainly gastrointestinal and, although severe in some patients, they are generally considered manageable. Efficacy of miltefosine for the treatment of *L. donovani* infections in Africa is still under study (Omollo *et al.* 2011), but seems to be low in populations with a high prevalence of HIV co-infection (Ritmeijer *et al.* 2001). Miltefosine was used for some years as the first line therapy for VL in parts of India, but the emergence of a relevant relapse rate has moved miltefosine down the line as an option mainly for combination therapy (Sundar *et al.* 2012; Rijal *et al.* 2013).

The antileishmanial activity of paromomycin, an aminoglycoside antibiotic, was first described in the 1960s (Neal *et al.* 1968). It was proposed as a topical agent for CL treatment (El-On *et al.* 1985). Paromomycin used by the parenteral route was later suggested as an alternative for VL (Chunge *et al.* 1990). Clinical trials carried on in India indicated that paromomycin was effective for the treatment of VL (Sundar *et al.* 2007). However, the same response was not found in East Africa, where trials demonstrated geographical heterogeneity and

Table 1. Summary of current antileishmanial drugs

Drug	2D structure	Molecular formula	Molecular weight (g mol ⁻¹)
			
Meglumine antimoniate		C ₇ H ₁₈ NO ₈ Sb	366
Sodium stibogluconate		C ₁₂ H ₃₈ Na ₃ O ₂₆ Sb ₂	911
Amphotericin B		C ₄₇ H ₇₃ NO ₁₇	924
Miltefosine		C ₂₁ H ₄₆ NO ₄ P	407
Paromomycin		C ₂₃ H ₄₅ N ₅ O ₁₄	616
Pentamidine		C ₁₉ H ₂₄ N ₄ O ₂	340

lower cure rates after treatment with paromomycin than with antimonials (Hailu *et al.* 2010; Musa *et al.* 2010, 2012). On the other hand, shorter courses of the association of paromomycin and antimonial were shown to be as effective as the long course of antimonial alone (Musa *et al.* 2012;

Atia *et al.* 2015), representing an advance in terms of therapeutic options. This scheme of association is now part of the WHO recommendations in these areas.

Historically, CL treatment has also made use of pentavalent antimonials as a first choice drug

(Gonzalez *et al.* 2008, 2009). The toxicity and parenteral routes of administration, together with the duration of treatment stimulated the use of alternative routes and drugs to treat the cutaneous form of the disease. For example, in benign cases (one or few small lesions in non-critical body areas) caused by *L. major*, local options such as topical paromomycin, thermotherapy, cryotherapy or intralesional antimonials have been applied.

The heterogeneity of clinical pictures, disease progression, complications and response to treatment of the various aetiological agents of CL makes the choice of therapy much less homogeneous than for VL. Another complicating factor is that most clinical trials conducted so far on the treatment of CL and mucocutaneous leishmaniasis, including studies on antimonials as well as other agents, were not well designed and reported, leading to inconclusive results (Gonzalez *et al.* 2008, 2009).

While topical agents are applied to *L. major* infections, New World CL is generally more severe with the potential of metastasis or progression to severe forms (diffuse or disseminated as well as mucocutaneous leishmaniasis) (Jirmanus *et al.* 2012). In addition, the spontaneous cure rate is very low in Americas (Cota *et al.* 2016). For these reasons, exclusive local treatment is generally not recommended and systemic drugs are employed with a great degree of geographical variation, including drugs with very specific applications, such as ketoconazole (for *L. mexicana*) (WHO, 2010). The systemic drugs applicable are pentavalent antimonials, pentamidine, amphotericin B and miltefosine. Notwithstanding, antimonials are still the first line treatment for CL in most leishmaniasis-endemic countries. Meglumine antimoniate is still used as first line option to treat CL in Brazil, for example, where even with more extensive schemes of antimonial treatment, recent studies have demonstrated a failure rate of over 40% in patients with tegumentary leishmaniasis in different geographic areas (Machado *et al.* 2010; Chrusciak-Talhari *et al.* 2011; Neves *et al.* 2011).

The use of amphotericin B in CL is generally reserved as a second or third option after treatment failure, mainly due to toxicity.

Pentamidine was synthesized at the end of the 1930s, as part of a diamidine group and as a synthalin analogue that showed activity against *Trypanosoma*. The demonstration of activity against *Leishmania* led to its use for the treatment of VL in India in the early 1940s (Bray *et al.* 2003). The high frequency of toxic effects, as for example, cardiotoxicity and metabolic disturbances, made pentamidine a forgotten option until the emergence of resistance against antimonials. However, the efficacy in the treatment of VL patients was unsatisfactory and a high number of relapses was noted (Jha *et al.* 1991), suggesting that resistance against pentamidine could be

easily selected. Pentamidine has found its niche in the treatment of infections in areas of South America where *L. guyanensis* is more prevalent (Van Der Meide *et al.* 2009).

The application of miltefosine in the treatment of CL is not yet completely established. The susceptibility of cutaneous *Leishmania* species to miltefosine is lower as compared with the susceptibility of *L. donovani* (Escobar *et al.* 2002). Clinical trials performed in Colombia and Nicaragua showed that miltefosine's efficacy was variable, and seemed to correlate not only with the *Leishmania* species, but also with geographical heterogeneity within the same species (Soto and Berman, 2006). The drug is approved for the treatment of CL in Colombia. It has been tried in Brazil in two studies that showed efficacy of about 70% for miltefosine in CL patients (Machado *et al.* 2010; Chrusciak-Talhari *et al.* 2011). Miltefosine was also shown to be non-inferior to pentavalent antimonial for the treatment of CL caused by *L. major* in Iran (Mohebbi *et al.* 2007).

Paromomycin was initially reported as a potential topical drug in animal models (El-On *et al.* 1984) and tested for CL treatment as a topical agent with variable results (Kim *et al.* 2009). Topical treatment of Old World CL with paromomycin was generally more effective than placebo and equivalent to intralesional pentavalent antimonials (El-On *et al.* 1992; Ben Salah *et al.* 2013). The association of paromomycin with methylbenzethonium chloride led to an increase in side-effects. Against New World species, topical paromomycin was inferior to pentavalent antimonials (Soto *et al.* 1998; Armijos *et al.* 2004).

All therapeutic alternatives mentioned above present considerable toxicity, high cost and, with the exception of miltefosine, have to be administered by the parenteral route. Furthermore, the risk or demonstration of parasite resistance has become a major concern.

DRUG RESISTANCE

Drug resistance is a phenotype of decreased susceptibility to a given drug acquired after drug selection. In the absence of previous exposure to drugs, natural variation in drug susceptibility does occur amongst species and clinical isolates of *Leishmania*. Reports of proven parasite resistance originated mainly from areas of anthroponotic transmission, such as the Indian subcontinent (Sundar, 2001). In endemic regions of zoonotic transmission, there is no definite demonstration of resistance yet. However, the recent disease urbanization may eventually change this scenario, leading to the emergence of drug resistant parasites in these regions (Harhay *et al.* 2011). Another serious concern is the increased number of HIV–leishmaniasis co-infected patients. In general, these patients respond poorly to the

treatment and exhibit high relapse rates, representing a potential source for the emergence of drug-resistant parasites (Molina *et al.* 2003).

A progressive decrease in the efficacy of pentavalent antimonials was described initially in VL patients. In the Northeast of India, where 85–95% of the VL patients were cured after treatment with antimony in 1981, therapeutic failures reached 60–70% by 1995 (Olliaro *et al.* 2005). Acquired resistance was confirmed in clinical isolates of *L. donovani* from this endemic region that were shown to be 3-fold less susceptible *in vitro* than isolates from patients who did respond to chemotherapy (Lira *et al.* 1999). Currently, the drug is not in use in the district of Bihar, India and in parts of Nepal and Bangladesh, due to the spread of antimony resistance. The contamination of drinking water with arsenic in Bihar has been implicated as an additional factor associated with the development of antimonial resistance (Perry *et al.* 2015).

In other VL endemic regions, lack of response to antimony does occur but detailed mapping of therapeutic failures is missing. *Leishmania infantum* isolates from dogs treated with antimony were shown to present decreased susceptibility to antimonials (Gramiccia *et al.* 1992). On the other hand, variability in clinical response can also be explained by intrinsic differences in the sensitivity of *Leishmania* species and strains to antimonials (Berman *et al.* 1982; Navin *et al.* 1992).

Studies performed in the laboratory have demonstrated that the parasite is easily selected *in vitro* for antimony resistance by drug pressure (Papadopoulou *et al.* 1994; Haimeur *et al.* 2000; Do Monte-Neto *et al.* 2011; Monte-Neto *et al.* 2015). The main drug resistance mechanisms have been correlated with reduction of drug concentration within the parasite, either by a decreased uptake mediated by the aquaglyceroporin AQP1, the primary route of antimony entry (Gourbal *et al.* 2004) or by increased efflux of the drug mediated by the ABC transporter ABCC3 (also known as MRPA) (Legare *et al.* 2001). Antimony resistant lines also present increased levels of thiols (cysteine, glutathione and trypanothione) due to overexpression/amplification of genes involved in the synthesis of glutathione and polyamines, the components of trypanothione, the main intracellular thiol in *Leishmania* (Mukhopadhyay *et al.* 1996; Grondin *et al.* 1997; Legare *et al.* 1997; Guimond *et al.* 2003; Do Monte-Neto *et al.* 2011). Interestingly, several of the mechanisms associated with antimony resistance *in vitro* were found in *L. donovani* clinical isolates. For these parasites, susceptibility *in vitro* of intracellular amastigotes correlated well with the clinical response (Mukherjee *et al.* 2007).

Resistance to antimony can also arise from inhibition of drug reduction or inactivation of the active drug form (Croft *et al.* 2006). Pentavalent antimony

is a prodrug that requires conversion to trivalent antimony to be active against the parasite. Two enzymes mediate this enzymatic reduction: a thiol-dependent reductase (TDR1) and an arsenate reductase (ACR2) (Denton *et al.* 2004; Zhou *et al.* 2004). The conversion to the active form is mediated by the host and by intracellular amastigotes, but not by promastigotes (Roberts and Rainey, 1993; Shaked-Mishan *et al.* 2001).

In addition, other molecular markers have been associated with antimony resistance by functional cloning, such as ABC transporters, phosphatases and hypothetical proteins (Choudhury *et al.* 2008; Manzano *et al.* 2013; Nuhs *et al.* 2014; Gazanion *et al.* 2016; Perea *et al.* 2016). However, their role in clinical resistance and treatment failure is still unknown.

Amphotericin is a polyene antibiotic that targets the main membrane sterol of the parasite, ergosterol. The drug has been in use, although not as a first-line option in the treatment of leishmaniasis for decades, and, until recently, without indication that clinical resistance was emerging, even when patients were exposed to multiple courses of treatment (Lachaud *et al.* 2009). However, lack of response to treatment with amphotericin B has now been reported in India, where this drug has become the first-line option in areas where refractoriness to antimony is widespread (Purkait *et al.* 2012, 2015).

Leishmania is not easily selected *in vitro* by amphotericin pressure. However, stepwise drug increase was applied successfully to select *Leishmania tarentolae* and *L. donovani* amphotericin resistant lines (Mbongo *et al.* 1998; Singh *et al.* 2001). These parasites exhibited gene amplification (Singh *et al.* 2001) and changes in drug-binding affinity to the plasma membrane as a result of a modified sterol composition (Mbongo *et al.* 1998). Recently, a screening method termed Cos-Seq was described employing functional cloning coupled to next-generation sequencing that allows the identification of potential drug targets and drug resistance genes (Gazanion *et al.* 2016). This strategy led to the identification of a hypothetical protein that contains transmembrane domains and a secretory signal peptide responsible for mediating amphotericin B resistance in promastigotes (Gazanion *et al.* 2016).

After its approval for VL treatment in India (Sundar *et al.* 2002) miltefosine replaced antimonials and was used as first-line therapy in northeastern India. However, an increase in the number of relapses was noted after a few years (Sundar *et al.* 2012; Rijal *et al.* 2013) affecting as many as 20% of patients infected with *L. donovani* 6–12 months after the end of treatment. The parasites isolated from relapsing patients did not present decreased drug susceptibility *in vitro* (Prajapati *et al.* 2013; Rijal *et al.* 2013), suggesting that the reduced clinical efficacy was related to other factors such as selection

of parasites with increased virulence/infectivity or lower exposure to the drug due to heterogeneous pharmacokinetics (Rai *et al.* 2013; Dorlo *et al.* 2014).

On the other hand, parasite resistance to miltefosine has been described in clinical isolates of *L. infantum* from a HIV-coinfected patient (Cojean *et al.* 2012) and *L. panamensis*, both rescued after treatment (Obonaga *et al.* 2014).

Miltefosine interferes in cell membrane composition by inhibiting phospholipid metabolism and affecting phosphatidylcholine and phosphatidylethanolamine synthesis due to a decrease in intracellular choline (Rakotomanga *et al.* 2007). Parasites treated with miltefosine also present a significant reduction in mitochondrial membrane potential and in cytochrome-c oxidase activity (Santa-Rita *et al.* 2004; Luque-Ortega and Rivas, 2007). The drug binds to the outer leaflet of the plasma membrane and is internalized by the endocytic pathway or by a flippase activity mediated by the miltefosine transporter (MT) and its non-catalytic subunit Ros3. This MT–Ros3 complex is responsible for phosphocoline accumulation in an ATP-dependent process (Perez-Victoria *et al.* 2003, 2006b; Coelho *et al.* 2014). Miltefosine is eliminated by exocytosis or by a floppase activity that may be mediated by members of ABC transporter subfamilies ABCB and ABCG (Perez-Victoria *et al.* 2001; Castanys-Munoz *et al.* 2007, 2008).

Susceptibility to miltefosine *in vitro* is intrinsically variable in different species and clinical isolates of *Leishmania* (Yardley *et al.* 2005; Coelho *et al.* 2014; Fernandez *et al.* 2014; Obonaga *et al.* 2014). This differential susceptibility among species and isolates may be explained by variations in the activity and substrate specificity of MT–Ros3 machinery, rate of cell division, biochemical targets, drug metabolism and biochemical composition of the plasma membrane (Perez-Victoria *et al.* 2006b; Sanchez-Canete *et al.* 2009). On the other hand, recent studies have shown an absence of correlation between miltefosine susceptibility *in vitro* and treatment outcome, indicating that this variation *in vitro* may not affect clinical efficacy (Rijal *et al.* 2013; Hendrickx *et al.* 2015).

Resistance to miltefosine is easily selected *in vitro* by stepwise increase in drug concentrations (Seifert *et al.* 2003; Coelho *et al.* 2012, 2014; Obonaga *et al.* 2014) or by chemical mutagenesis (Perez-Victoria *et al.* 2003; Coelho *et al.* 2015). In general, mechanisms of miltefosine resistance are associated with a defect in drug internalization mediated by the machinery MT–Ros3 (Perez-Victoria *et al.* 2006b). Mutations in the *MT* and *Ros3* genes have been found after selection with miltefosine, with a higher frequency of mutations in the *MT* gene (Perez-Victoria *et al.* 2003, 2006a; Coelho *et al.* 2012, 2014; Kulshrestha *et al.* 2014; Mondelaers *et al.* 2016; Shaw *et al.* 2016). Interestingly, the

resistance phenotype mediated by MT inactivation persists in animal models of visceral and cutaneous disease (Seifert *et al.* 2007; Coelho *et al.* 2014), indicating that MT activity is essential for miltefosine efficacy *in vivo*.

Pentamidine was not used extensively for VL treatment, due to failure and toxicity, but it has its place in the treatment of CL. Parasites resistant to pentamidine are rapidly obtained *in vitro* for several *Leishmania* species (Ellenberger and Beverley, 1989; Sereno and Lemesre, 1997; Basselin *et al.* 2002; Coelho *et al.* 2008). Pentamidine resistant lines display changes in intracellular concentrations of arginine and polyamines, reduction in pentamidine accumulation in the mitochondria and increased efflux of the drug (Basselin *et al.* 2002). It is possible that the ABC transporter PRP1, isolated previously by functional cloning, mediates this efflux (Coelho *et al.* 2003). In addition to PRP1, functional cloning strategies using Cos-Seq identified a hypothetical protein that mediates low level of resistance to pentamidine in promastigotes (Gazanion *et al.* 2016). It would be interesting to evaluate whether parasites isolated from unresponsive cases are associated with overexpression of these genes.

Paromomycin is an aminoglycoside antibiotic that targets protein synthesis in the parasite (Maarouf *et al.* 1995; Fernandez *et al.* 2011). The drug binds to the ribosome, specifically to the ribosomal A-site (Shalev *et al.* 2013). Paromomycin also induces alterations in membrane fluidity and lipid metabolism and affects mitochondrial activity (Maarouf *et al.* 1997). Resistant parasites can be selected *in vitro* and these lines show a decreased drug accumulation, although the protein/transporter involved is still unknown (Jhingran *et al.* 2009; Bhandari *et al.* 2014). Mutations in the small subunit ribosomal RNA gene were found in paromomycin resistant mutants of *Escherichia coli* and *Tetrahymena* (Fong *et al.* 1994), but not in *Leishmania*-resistant lines, suggesting that the mechanisms leading to paromomycin resistance are variable. So far, there is only one paromomycin resistance gene identified in *Leishmania* (Gazanion *et al.* 2016). It encodes a hypothetical protein that contains a leucine-rich repeat domain that also confers resistance to pentamidine (Gazanion *et al.* 2016). The mechanism of multiple drug resistance mediated by this protein is still uncertain. Clinical resistance to paromomycin has not yet been reported, fact perhaps explained by the recent introduction of the drug for the chemotherapy of the disease.

Heterogeneity in paromomycin susceptibility has been observed in different species and clinical isolates of the parasite (Neal *et al.* 1995; Kulshrestha *et al.* 2011; Prajapati *et al.* 2012). Whether these differences detected *in vitro* affect treatment response in the field remains to be determined.

In fact, studies to determine the correlation between the various molecular markers of drug

resistance described *in vitro* and treatment failure/clinical resistance need to be expanded. The problem of treatment failure is a complex one given that it can also be due to mishandling of antileishmanial drugs, individual variability in the susceptibility of *Leishmania* species and isolates, pharmacokinetics and drug–host immune response interaction (Croft *et al.* 2006). An additional factor that may also affect clinical efficacy and lead to treatment failure is the presence of *Leishmania* RNA virus 1 (LRV-1) in parasites of the *Viannia* subgenus. Recent data indicated that the virus may subvert the host immune response and affect clinical response to drugs (Adaui *et al.* 2015; Bourreau *et al.* 2016).

Given that resistance to all antileishmanial drugs has been detected, at least in the laboratory, there is now a consensus on the necessity of adopting combination therapy as the preferred treatment option, especially for the life-threatening VL (WHO, 2012).

COMBINATION THERAPY

Combination therapy consists of using two or more drugs with synergistic or additive effects and distinct mechanisms of action with the aim of increasing the spectrum of activity and therapeutic efficacy. Ideally, combination therapy will reduce the overall dose of drugs and treatment duration, resulting in lower toxicity and higher compliance. It can also reduce costs and therefore the burden on the health system (Van Griensven *et al.* 2010; WHO, 2010). Combination therapy can also help delaying the selection of drug-resistant parasites, prolonging the effective life of available medicines, as has been reported in diseases such as tuberculosis, malaria and AIDS (Vandamme *et al.* 1999; Kremsner and Krishna, 2004; Van Griensven *et al.* 2010; Mitchison and Davies, 2012).

The implementation of combination therapy for leishmaniasis requires the determination of the best combination schemes and their efficacy in clinical settings. Efficacy studies reported or in progress have employed combinations of drugs already available – pentavalent antimonials, amphotericin B, miltefosine and paromomycin – and were performed mainly with VL patients in India and Africa.

Ongoing initiatives have focused mainly on two combination schemes: pentavalent antimonial associated with paromomycin and amphotericin B plus miltefosine. The first strategy is being applied mainly in Africa, given the high incidence of antimonial resistance in Asia.

Initial clinical trials testing the interaction between pentavalent antimonial and paromomycin date from the early 1990s, were performed in Kenya, India and Sudan, and demonstrated an enhanced overall efficacy and/or reduced treatment duration for the combination as compared with

antimonial alone (Chunge *et al.* 1990; Thakur *et al.* 1992; Seaman *et al.* 1993). A combination of sodium stibogluconate combined with paromomycin for 17 days was shown to be effective in southern Sudan with higher survival and initial cure rates in patients treated with the combination than with sodium stibogluconate alone (Melaku *et al.* 2007). A phase 3 trial in Ethiopia, Kenya, Sudan and Uganda confirmed that the use of sodium stibogluconate combined with paromomycin for 17 days was safe and non-inferior to the standard 30-day antimonial treatment (Musa *et al.* 2012).

Based on these data, WHO recommendations were changed in 2010 to advocate the use of sodium stibogluconate and paromomycin combination as the first-line treatment in East Africa (Ethiopia, Eritrea, Kenya, Somalia, Sudan and Uganda) and Yemen (WHO, 2010). A recent assessment of this treatment scheme indicated a cure rate of 86% at 6 months (Atia *et al.* 2015). However, it has also made clear that, although representing an improvement over past options given the reduction in hospitalization time, it still lacks in comprehensiveness: a considerable number of patients are excluded from this scheme because of underlying conditions, such as HIV co-infection, pregnancy, renal failure or due to disease severity, highlighting the necessity of the continued search for new drugs and treatment regimens to improve the handling of VL.

The interaction of amphotericin B and miltefosine has been investigated mainly in Indian VL patients. Phase 2 and 3 studies, performed in Bihar, India, have evaluated combinations of a single dose of liposomal amphotericin B followed by miltefosine given for 7, 10 or 14 days (Sundar *et al.* 2008). Other combination alternatives were also evaluated, such as single-dose liposomal amphotericin B plus a 10-day course of intramuscular paromomycin and the combination of miltefosine and paromomycin for 10 days (Sundar *et al.* 2011). All tested combinations were well tolerated and showed high efficacy with cure rates at 6–9 months follow-up higher than 95% and were therefore non-inferior to the standard treatment with amphotericin B in that endemic area.

A short course of amphotericin B combined with miltefosine is an attractive option for Indian VL due to the ease of administration, efficacy and tolerance. The reduction in time and amount of drug administered contribute to reduced toxicity and cost of therapy, besides protecting miltefosine from selection of drug resistant parasites.

Combination therapy is also strongly recommended for HIV/*Leishmania* co-infected patients (WHO, 2010). Visceral leishmaniasis in HIV co-infected patients represents a tremendous treatment challenge since patients that do respond to monotherapy present a high relapse rate. Furthermore, given the seriousness of the co-morbidities, high

mortality rates and increased antileishmanial drug toxicity are observed (Alvar *et al.* 2008). Antiretroviral treatment is a crucial measure associated with antileishmanial therapy. Protease inhibitors available for second-line treatment of HIV-positive patients have demonstrated antileishmanial activity *in vitro* (Kumar *et al.* 2010; Valdivieso *et al.* 2010; Santos *et al.* 2013; Van Griensven *et al.* 2013) and, pending clinical studies, might become valuable partners in combination therapy against leishmaniasis in these patients.

Unfortunately, there are still no evidence-based guidelines for the use of drug combinations in the treatment of these patients. A retrospective analysis of the treatment of HIV–*Leishmania* co-infected patients with a combination of liposomal amphotericin and miltefosine revealed the safety and efficacy of this scheme (Mahajan *et al.* 2015). A lower relapse rate was detected in these patients in comparison with previous data available for monotherapy with liposomal amphotericin (Burza *et al.* 2014). A phase 3 clinical trial aimed at evaluating the safety and efficacy of liposomal amphotericin and miltefosine combination in co-infected patients is ongoing in Ethiopia.

Secondary prophylaxis is adopted in some countries after primary treatment for leishmaniasis in AIDS patients, at least until the immune status of the patient can be considered minimally reconstituted. Even with ongoing prophylaxis, relapse rates can be very high (Diro *et al.* 2015). There are no data available on the use of drug combinations for secondary prophylaxis.

Current knowledge on combination therapy for CL is even more limited. The use of pentavalent antimonial combined with pentoxifylline, an inhibitor of tumour necrosis factor alpha, has proven effective against aggressive forms, such as mucocutaneous leishmaniasis (Lessa *et al.* 2001; Machado *et al.* 2007). This combination scheme was also effective in CL patients in Iran (Sadeghian and Nilforoushzadeh, 2006).

For severe forms of CL, the use of topical or local treatment is not advisable. However, the combination of a topical agent to a systemic drug may represent an interesting option and should be further investigated.

DRUG SCREENING

The need for new chemotherapeutic alternatives for leishmaniasis is evident. The Target Product Profile (TPP) proposed by DNDi (<http://www.dndi.org/diseases-projects/leishmaniasis>) includes consideration of: (a) activity against all *Leishmania* species, (b) efficacy against VL and CL, (c) efficacy with courses of treatment shorter than 14 days, (d) oral drug with single daily dose preferred, (e) safer than available treatment, (f) low cost per treatment and

(g) stability under conditions that can be achieved in target regions. It is also highly desirable that efficacy is maintained in immunosuppressed patients. Furthermore, it is desirable that new drugs are compatible for combination therapy.

Drug discovery projects have been undertaken by many laboratories using classical drug-to-target or target-to-drug strategies. Drug discovery processes and challenges applicable to infectious diseases have been recently reviewed (Lechartier *et al.* 2014; Manjunatha *et al.* 2015) and challenges specific to trypanosomatids have been also described in detail (Don *et al.* 2014; Reguera *et al.* 2014).

Screening of chemical libraries for the specific target inhibition or phenotypic outcomes, as well as drug repurposing have all been employed for leishmaniasis. Unbiased screening of chemical libraries makes use of phenotypic evaluations and has the potential of identifying new entities directed at novel targets (Annang *et al.* 2015; Kaiser *et al.* 2015a; Pena *et al.* 2015; Khare *et al.* 2016; Khraiwesh *et al.* 2016). The opposite strategy elects a target that has been deemed of importance for the parasite and ideally is absent in the host. Examples of targets that have been used for drug screening purposes are thymine reductase (Richardson *et al.* 2009; Pandey *et al.* 2015), N-myristoyltransferase (Bell *et al.* 2012; Rackham *et al.* 2015), inositol phosphorylceramide synthase (Mina *et al.* 2010), dipeptidylcarboxypeptidase (Gangwar *et al.* 2012), among others. Target-based drug screening has the disadvantage of not always translating into parasite killing or *in vivo* activity, as has been extensively demonstrated by target-based efforts. Therefore, a trend towards high-throughput phenotypic screening has been noted in the last years. Also, the introduction of high-content screening platforms emerged as a promising approach, allowing large libraries to be tested against *Leishmania* intracellular amastigotes (Siqueira-Neto *et al.* 2012; Forestier *et al.* 2015). This approach has several advantages including the differentiation between activity against the parasite or the host cell, and the possibility of identification of singular cell events that can guide the research on the drug's mode of action.

Another aspect that has been advanced into earlier stages of drug screening is the evaluation of pharmacokinetics and pharmacodynamics (Lin and Lu, 1997). Adequate properties are fundamental criteria if a positive hit is to achieve the status of a drug candidate. Bioavailability, drug metabolism, activity of metabolites and drug–drug interactions should be evaluated as soon as possible in the drug discovery process (Riley *et al.* 2004; Shearer *et al.* 2005; Li *et al.* 2013).

The use of amphotericin B and miltefosine for leishmaniasis treatment are examples of successful repurposing. Other active drugs that were or are

Table 2. Commonly used leishmaniasis experimental models employed for *in vivo* testing

Aetiological agent	Disease	Animal model	Observations	References
<i>L. donovani</i>	VL	Hamster		Manandhar <i>et al.</i> (2008), Prajapati <i>et al.</i> (2011)
<i>L. donovani</i>	VL	Mice	Can be self-healing	Croft <i>et al.</i> (1996), Gupta <i>et al.</i> (2015), Wyllie <i>et al.</i> (2012)
<i>L. infantum</i>	VL	Hamster		Fortin <i>et al.</i> (2012), Reimao <i>et al.</i> (2011), Sanchez-Brunete <i>et al.</i> (2004)
<i>L. major</i>	CL	Mice	Not all mice strains susceptible	El-On and Hamburger (1987), Nabors <i>et al.</i> (1995), Yardley and Croft (1997)
<i>L. amazonensis</i>	CL	Mice	Common mice strains very susceptible	Aguiar <i>et al.</i> (2010), Arruda <i>et al.</i> (2005, 2009), Miguel <i>et al.</i> (2009)
<i>L. braziliensis</i>	CL	Hamster		De Mello <i>et al.</i> (2015), Goncalves <i>et al.</i> (2005)
<i>L. braziliensis</i>	CL	Mice	Generally self-healing	Coelho <i>et al.</i> (2016), Miguel <i>et al.</i> (2009), Santos <i>et al.</i> (2014)

used for human or canine leishmaniasis, such as various azoles, pentoxifylline and allopurinol were all repurposed from other indications. Drug screening based on drug repurposing is still an active area of research in leishmaniasis. Examples include the demonstration of antileishmanial activity of protein kinase inhibitors (Sanderson *et al.* 2014), phosphoinositide-3-kinases (Diaz-Gonzalez *et al.* 2011) and of the anti-*Mycobacterium* drugs clofazimine and delamanid (Evans *et al.* 1989; Kaiser *et al.* 2015b; Patterson *et al.* 2016). Another example is the antileishmanial activity of the group of molecules named SERM (selective oestrogen receptor modulators), of which the best-studied example is tamoxifen.

Tamoxifen is uniformly active against all *Leishmania* species tested so far (Miguel *et al.* 2007, 2009) and against clinical isolates from CL and VL patients (Miguel *et al.* 2011). The mechanisms of the leishmanicidal activity have not been fully elucidated but do not depend on interaction with oestrogen receptors (Bonano *et al.* 2014). Amongst other effects, the drug reduces the acidification of the parasitophorous vacuoles that normally harbour a hydrolytic acid environment (Miguel *et al.* 2007).

Tamoxifen is used continuously for 5 years in breast cancer patients, with minimal side-effects in the short term (Gajdos and Jordan, 2002), and therefore should be safe for leishmaniasis treatment. Interestingly, all attempts to generate parasites resistant to tamoxifen *in vitro* were unsuccessful (Coelho *et al.* 2015), indicating that this might be an interesting molecule to use in combination therapy.

In vitro drug screening, whatever the strategy used, will produce candidates to new drugs that need to be tested *in vivo*, in suitable animal models.

ANIMAL MODELS

The definition of a relevant animal model should be based on the purpose of the test. In the case of animal models for drug development, it is to be expected that they should mimic natural infection and

exhibit pathology, immune response and clinical evolution comparable with the disease in humans. Ideally, drug pharmacokinetics and pharmacodynamics in the model should also be comparable with humans. The animals should also be easy to keep and handle and preferably not very expensive. One has to take into consideration that this is a chronic disease and long periods will be required to allow response to be noticed and confirmed.

The establishment of a good experimental model for leishmaniasis will depend on fixing quite a number of variables. The multiplicity of leishmaniasis clinical presentations presents the first challenge. Animal models of localized CL and of VL have been described (Table 2). However, we have not yet found good models for mucosal, diffuse or disseminated leishmaniasis.

The experimental design of an *in vivo* efficacy test for a candidate drug has to start from the choice of parasite and host species (and therefore of clinical disease) that will be used as a model. Within the parasite species chosen, we should also define which strain will be used, since even within the same species, virulence and behaviour can be variable, as well as response to treatment. Strains of *L. donovani* from India and Africa are good examples of this heterogeneity (Kauffmann *et al.* 2016). It is important to keep in mind that *Leishmania* parasites kept under *in vitro* conditions can lose their virulence.

As for the inoculum appropriate to mimic the natural infection, the ideal procedure would include sandfly transmission, given the impact of insect factors in the success of the infection (Sacks and Kamhawi, 2001; Aslan *et al.* 2013). However, that is not practical since phlebotomine colonies are extremely difficult to keep. Instead, one frequently settles for using metacyclic parasites, which are the infective form inoculated by the sandfly. Separation of metacyclic from procyclic promastigotes is well established for some species (Da Silva and Sacks, 1987) but, for most

Leishmania species, the best that can be achieved is to enrich the population in metacyclics by density gradient (Spath and Beverley, 2001).

The number of parasites used in the inoculum has an impact on the development of disease. Generally but within limits, the higher the number of parasites used in the inoculum, the shorter is the incubation period. Higher numbers in the inoculum may also be associated with a more disseminated pattern of disease, with visceralization of cutaneous species (Ribeiro-Romao *et al.* 2014).

Also, parasites inoculated at different sites in the same host species may behave distinctly. For example, *L. amazonensis* inoculation in C57Bl/6 mice in the rump skin or in the footpad results in the development of lesions with different characteristics: while rump inoculation led to the development of a large ulcerated lesion, the same number of parasites inoculated in the footpad led to only minimal oedema (Felizardo *et al.* 2012). For VL models, inoculation in the venous territory is preferable to establish infection successfully (Moreira *et al.* 2016).

The time to start the treatment is the next question. Some investigators choose to perform proof-of-concept tests of drug efficacy treating inoculated animals immediately after (sometimes even prior to) parasite inoculation. These are very artificial conditions and would never be encountered in clinical settings. In our opinion, the infection has to be fully established before treatment is initiated, either with lesions clearly observable in the case of CL models, or at times when the infection has been demonstrated to be fully established in VL models. Lately, with the advent of parasites expressing reporter proteins, it is possible to make use of *in vivo* imaging to establish the parasite burden of subject animals before initiating treatment.

Dose, route and frequency of administration, as well as duration of treatment have to be carefully chosen. These decisions would be ideally made based on solubility, pharmacokinetics and pharmacodynamic properties of the test drug. Unfortunately, these data are not always available and are not easily obtained for a new compound. The next best thing, in our opinion, is to determine the maximal tolerated dose that can be used as a starting point in a proof-of-concept testing.

Parameters of efficacy have to be determined. The inoculation of some *L. braziliensis* strains into BALB/c mice is followed by the appearance of a lesion that heals spontaneously in some weeks (De Moura *et al.* 2005). In these circumstances, the only possible measure of efficacy is to compare the development of lesions in treated and untreated animals at the point of maximal lesion size and parasite burden for control animals (Miguel *et al.* 2009). On the other hand, disease established by inoculation of *L. infantum chagasi* in hamsters develops into a chronic disease that ultimately leads to death (Reimao *et al.*

2015). In this model, a possible end-point could be survival. The method to evaluate efficacy is also variable depending on the model. Lesion size may be useful for CL evaluation, but it has to be taken carefully. For example, *L. amazonensis* lesions in BALB/c mice increase progressively in size until they ulcerate. From that point onwards, the size of the lesion, measured as footpad thickness, for example, decreases. That could be mistaken as a sign of drug-driven improvement if only the lesion size is considered.

The most strict criterion to determine drug efficacy is the evaluation of parasite burden. Classically, this was done in leishmaniasis by limiting dilution (Lima *et al.* 1997), a cumbersome technique. More recently, real-time PCR is being employed as an alternative to limiting dilution (Nicolas *et al.* 2002; Srivastava *et al.* 2013). Both limiting dilution and qPCR require animal sacrifice since tissue is obtained and processed for parasite quantification. Bioimaging has brought several improvements to this scenario. Sensitive reporters have been applied successfully to *Leishmania* detection, allowing the determination of parasite burden before treatment is initiated and during the follow up of the same animals. Good correlations have been observed between bioimaging and the most traditional techniques (Lang *et al.* 2005; Michel *et al.* 2011; Reimao *et al.* 2013, 2015).

A sustained response to treatment is an important characteristic of a good candidate drug and that should be evaluated through a thorough post-treatment follow-up of treated animals.

CONCLUSIONS/FUTURE DIRECTIONS

Shortcomings in leishmaniasis chemotherapy are evident. Given the reduced clinical success rates, parasite resistance, toxicity and/or cost of current drugs, new effective alternatives are clearly necessary. Due to the wide variety of aetiological agents, pre-clinical studies should include animal models representative of the different forms of the disease. It is very important to make sound choices on experimental models to test drug candidates. General criteria for efficacy evaluation on pre-clinical studies should be agreed upon, so that studies performed in different laboratories can be compared. The development of methods for species-specific diagnosis easily performed and widely accessible would allow the investigation of better protocols to treat specific forms of CL. Combination therapy is clearly a necessity and extensive clinical tests of combination schemes should pave the way to fundamentally change leishmaniasis therapy.

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REFERENCES

- Adaui, V., Lye, L.F., Akopyants, N.S., Zimic, M., Llanos-Cuentas, A., Garcia, L., Maes, I., De Doncker, S., Dobson, D.E., Arevalo, J., Dujardin, J. C. and Beverley, S. M. (2015). Association of the endobiont double-stranded RNA virus LRV1 with treatment failure for human leishmaniasis caused by *Leishmania braziliensis* in Peru and Bolivia. *Journal of Infectious Diseases* **213**, 112–121.
- Aguiar, M. G., Pereira, A. M., Fernandes, A. P. and Ferreira, L. A. (2010). Reductions in skin and systemic parasite burdens as a combined effect of topical paromomycin and oral miltefosine treatment of mice experimentally infected with *Leishmania (Leishmania) amazonensis*. *Antimicrobial Agents Chemotherapy* **54**, 4699–4704.
- Alawieh, A., Musharrafieh, U., Jaber, A., Berry, A., Ghosn, N. and Bizri, A. R. (2014). Revisiting leishmaniasis in the time of war: the Syrian conflict and the Lebanese outbreak. *International Journal of Infectious Diseases* **29**, 115–119.
- Alvar, J., Aparicio, P., Aseffa, A., Den Boer, M., Canavate, C., Dedet, J. P., Gradoni, L., Ter Horst, R., Lopez-Velez, R. and Moreno, J. (2008). The relationship between leishmaniasis and AIDS: the second 10 years. *Clinical Microbiology Reviews* **21**, 334–359.
- Alvar, J., Velez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J. and Den Boer, M. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE* **7**, e35671.
- Annang, F., Perez-Moreno, G., Garcia-Hernandez, R., Cordon-Obras, C., Martin, J., Tormo, J. R., Rodriguez, L., De Pedro, N., Gomez-Perez, V., Valente, M., Reyes, F., Genilloud, O., Vicente, F., Castanys, S., Ruiz-Perez, L. M., Navarro, M., Gamarro, F. and Gonzalez-Pacanoska, D. (2015). High-throughput screening platform for natural product-based drug discovery against 3 neglected tropical diseases: human African trypanosomiasis, leishmaniasis, and Chagas disease. *Journal of Biomolecular Screening* **20**, 82–91.
- Armijos, R. X., Weigel, M. M., Calvopina, M., Mancheno, M. and Rodriguez, R. (2004). Comparison of the effectiveness of two topical paromomycin treatments versus meglumine antimoniate for New World cutaneous leishmaniasis. *Acta Tropica* **91**, 153–160.
- Arruda, D., D'alexandri, F., Katzin, A. and Uliana, S. (2005). Antileishmanial activity of the terpene nerolidol. *Antimicrobial Agents and Chemotherapy* **49**, 1679–1687.
- Arruda, D. C., Miguel, D. C., Yokoyama-Yasunaka, J. K., Katzin, A. M. and Uliana, S. R. (2009). Inhibitory activity of limonene against *Leishmania* parasites *in vitro* and *in vivo*. *Biomedicine and Pharmacotherapy* **63**, 643–649.
- Aslan, H., Dey, R., Meneses, C., Castrovinci, P., Jeronimo, S. M., Oliva, G., Fischer, L., Duncan, R. C., Nakhasi, H. L., Valenzuela, J. G. and Kamhawi, S. (2013). A new model of progressive visceral leishmaniasis in hamsters by natural transmission via bites of vector sand flies. *Journal of Infectious Diseases* **207**, 1328–1338.
- Atia, A. M., Mumina, A., Tayler-Smith, K., Boule, P., Alcoba, G., Elhag, M. S., Alnour, M., Shah, S., Chappuis, F., Van Griensven, J. and Zachariah, R. (2015). Sodium stibogluconate and paromomycin for treating visceral leishmaniasis under routine conditions in eastern Sudan. *Tropical Medicine and International Health* **20**, 1674–1684.
- Balasegaram, M., Ritmeijer, K., Lima, M. A., Burza, S., Ortiz Genovese, G., Milani, B., Gaspani, S., Potet, J. and Chappuis, F. (2012). Liposomal amphotericin B as a treatment for human leishmaniasis. *Expert Opinion on Emerging Drugs* **17**, 493–510.
- Basselin, M., Denise, H., Coombs, G. H. and Barrett, M. P. (2002). Resistance to pentamidine in *Leishmania mexicana* involves exclusion of the drug from the mitochondrion. *Antimicrobial Agents and Chemotherapy* **46**, 3731–3738.
- Bell, A. S., Mills, J. E., Williams, G. P., Brannigan, J. A., Wilkinson, A. J., Parkinson, T., Leatherbarrow, R. J., Tate, E. W., Holder, A. A. and Smith, D. F. (2012). Selective inhibitors of protozoan protein N-myristoyltransferases as starting points for tropical disease medicinal chemistry programs. *PLoS Neglected Tropical Diseases* **6**, e1625.
- Ben Salah, A., Ben Messaoud, N., Guedri, E., Zaatour, A., Ben Alaya, N., Bettaieb, J., Gharbi, A., Belhadj Hamida, N., Boukthir, A., Chlif, S., Abdelhamid, K., El Ahmadi, Z., Louzir, H., Mokni, M., Morizot, G., Buffet, P., Smith, P. L., Kopydlowski, K. M., Kreishman-Deitrick, M., Smith, K. S., Nielsen, C. J., Ullman, D. R., Norwood, J. A., Thorne, G. D., Mccarthy, W. F., Adams, R. C., Rice, R. M., Tang, D., Berman, J., Ransom, J. *et al.* (2013). Topical paromomycin with or without gentamicin for cutaneous leishmaniasis. *New England Journal of Medicine* **368**, 524–532.
- Berman, J. D., Chulay, J. D., Hendricks, L. D. and Oster, C. N. (1982). Susceptibility of clinically sensitive and resistant *Leishmania* to pentavalent antimony *in vitro*. *American Journal of Tropical Medicine and Hygiene* **31**, 459–465.
- Berry, I. and Berrang-Ford, L. (2016). Leishmaniasis, conflict, and political terror: a spatio-temporal analysis. *Social Science and Medicine* **167**, 140–149. doi:10.1016/j.socscimed.2016.04.038.
- Bhandari, V., Sundar, S., Dujardin, J. C. and Salotra, P. (2014). Elucidation of cellular mechanisms involved in experimental paromomycin resistance in *Leishmania donovani*. *Antimicrobial Agents and Chemotherapy* **58**, 2580–2585.
- Bonano, V. I., Yokoyama-Yasunaka, J. K., Miguel, D. C., Jones, S. A., Dodge, J. A. and Uliana, S. R. (2014). Discovery of synthetic *Leishmania* inhibitors by screening of a 2-arylbenzothiothiophene library. *Chemical Biology & Drug Design* **83**, 289–296.
- Botero Aguirre, J. P. and Restrepo Hamid, A. M. (2015). Amphotericin B deoxycholate versus liposomal amphotericin B: effects on kidney function. *Cochrane Database of Systematic Reviews*, 11. Art. No.: CD010481. doi: 10.1002/14651858.CD010481.pub2.
- Bourreau, E., Ginouves, M., Prevot, G., Hartley, M. A., Gangneux, J. P., Robert-Gangneux, F., Dufour, J., Sainte-Marie, D., Bertolotti, A., Pralongo, F., Martin, R., Schutz, F., Couppie, P., Fasel, N. and Ronet, C. (2016). Presence of *Leishmania* RNA virus 1 in *Leishmania guyanensis* increases the risk of first-line treatment failure and symptomatic relapse. *Journal of Infectious Diseases* **213**, 105–111.
- Bray, P. G., Barrett, M. P., Ward, S. A. and de Koning, H. P. (2003). Pentamidine uptake and resistance in pathogenic protozoa: past, present and future. *Trends Parasitol* **19**, 232–239.
- Burza, S., Mahajan, R., Sinha, P. K., Van Griensven, J., Pandey, K., Lima, M. A., Sanz, M. G., Sunyoto, T., Kumar, S., Mitra, G., Kumar, R., Verma, N. and Das, P. (2014). Visceral leishmaniasis and HIV co-infection in Bihar, India: long-term effectiveness and treatment outcomes with liposomal amphotericin B (AmBisome). *PLoS Neglected Tropical Diseases* **8**, e3053.
- Castanys-Munoz, E., Alder-Baerens, N., Pomorski, T., Gamarro, F. and Castanys, S. (2007). A novel ATP-binding cassette transporter from *Leishmania* is involved in transport of phosphatidylcholine analogues and resistance to alkyl-phospholipids. *Molecular Microbiology* **64**, 1141–1153.
- Castanys-Munoz, E., Perez-Victoria, J. M., Gamarro, F. and Castanys, S. (2008). Characterization of an ABCG-like transporter from the protozoan parasite *Leishmania* with a role in drug resistance and trans-bilayer lipid movement. *Antimicrobial Agents and Chemotherapy* **52**, 3573–3579.
- Choudhury, K., Zander, D., Kube, M., Reinhardt, R. and Clos, J. (2008). Identification of a *Leishmania infantum* gene mediating resistance to miltefosine and SbIII. *International Journal of Parasitology* **38**, 1411–1423.
- Chrusciak-Talhari, A., Dietze, R., Chrusciak Talhari, C., Da Silva, R. M., Gadelha Yamashita, E. P., De Oliveira Penna, G., Lima Machado, P. R. and Talhari, S. (2011). Randomized controlled clinical trial to access efficacy and safety of miltefosine in the treatment of cutaneous leishmaniasis Caused by *Leishmania (Viannia) guyanensis* in Manaus, Brazil. *American Journal of Tropical Medicine and Hygiene* **84**, 255–260.
- Chunge, C. N., Owate, J., Pamba, H. O. and Donno, L. (1990). Treatment of visceral leishmaniasis in Kenya by aminosidine alone or combined with sodium stibogluconate. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 221–225.
- Coelho, A. C., Beverley, S. M. and Cotrim, P. C. (2003). Functional genetic identification of PRP1, an ABC transporter superfamily member conferring pentamidine resistance in *Leishmania major*. *Molecular Biochemical Parasitology* **130**, 83–90.
- Coelho, A. C., Gentil, L. G., Da Silveira, J. F. and Cotrim, P. C. (2008). Characterization of *Leishmania (Leishmania) amazonensis* promastigotes resistant to pentamidine. *Experimental Parasitology* **120**, 98–102.
- Coelho, A. C., Boisvert, S., Mukherjee, A., Leprohon, P., Corbeil, J. and Ouellette, M. (2012). Multiple mutations in heterogeneous miltefosine-resistant *Leishmania major* population as determined by whole genome sequencing. *PLoS Neglected Tropical Diseases* **6**, e1512.

- Coelho, A. C., Trincon, C. T., Costa, C. H. and Uliana, S. R. (2014). *In vitro* and *in vivo* miltefosine susceptibility of a *Leishmania amazonensis* isolate from a patient with diffuse cutaneous leishmaniasis. *PLoS Neglected Tropical Diseases* **8**, e2999.
- Coelho, A. C., Trincon, C. T., Senra, L., Yokoyama-Yasunaka, J. K. and Uliana, S. R. (2015). *Leishmania* is not prone to develop resistance to tamoxifen. *International Journal of Parasitology: Drugs and Drug Resistance* **5**, 77–83.
- Coelho, A. C., Oliveira, J. C., Espada, C. R., Reimao, J. Q., Trincon, C. T. and Uliana, S. R. (2016). A luciferase-expressing *Leishmania braziliensis* line that leads to sustained skin lesions in BALB/c mice and allows monitoring of miltefosine treatment outcome. *PLoS Neglected Tropical Diseases* **10**, e0004660.
- Cojean, S., Houze, S., Haouchine, D., Huteau, F., Lariven, S., Hubert, V., Michard, F., Bories, C., Pralong, F., Le Bras, J., Loiseau, P. M. and Matheron, S. (2012). *Leishmania* resistance to miltefosine associated with genetic marker. *Emerging Infectious Diseases* **18**, 704–706.
- Collin, S., Davidson, R., Ritmeijer, K., Keus, K., Melaku, Y., Kipngetich, S. and Davies, C. (2004). Conflict and kala-azar: determinants of adverse outcomes of kala-azar among patients in southern Sudan. *Clinical Infectious Diseases* **38**, 612–619.
- Cota, G. F., De Sousa, M. R., Fereguetti, T. O., Saleme, P. S., Alvaris, T. K. and Rabello, A. (2016). The cure rate after placebo or no therapy in American Cutaneous Leishmaniasis: a systematic review and meta-analysis. *PLoS ONE* **11**, e0149697.
- Croft, S. L., Neal, R. A., Pendergast, W. and Chan, J. H. (1987). The activity of alkyl phosphorylcholines and related derivatives against *Leishmania donovani*. *Biochemical Pharmacology* **36**, 2633–2636.
- Croft, S. L., Snowdon, D. and Yardley, V. (1996). The activities of four anticancer alkyllysophospholipids against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei*. *Journal of Antimicrobial Chemotherapy* **38**, 1041–1047.
- Croft, S. L., Sundar, S. and Fairlamb, A. H. (2006). Drug resistance in leishmaniasis. *Clinical Microbiology Reviews* **19**, 111–126.
- Da Silva, R. and Sacks, D. L. (1987). Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Infection and Immunity* **55**, 2802–2806.
- De Mello, T. F., Cardoso, B. M., Lopes, S. N., Bitencourt, H. R., Voltarelli, E. M., Hernandes, L., Aristides, S. M., Lonardoni, M. V. and Silveira, T. G. (2015). Activity of synthetic chalcones in hamsters experimentally infected with *Leishmania (Viannia) braziliensis*. *Parasitology Research* **114**, 3587–3600.
- De Moura, T. R., Novais, F. O., Oliveira, F., Clarencio, J., Noronha, A., Barral, A., Brodskyn, C. and De Oliveira, C. I. (2005). Toward a novel experimental model of infection to study American cutaneous leishmaniasis caused by *Leishmania braziliensis*. *Infection and Immunity* **73**, 5827–5834.
- Denton, H., Mcgregor, J. C. and Coombs, G. H. (2004). Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1. *The Biochemical Journal* **381**, 405–412.
- Diaz-Gonzalez, R., Kuhlmann, F. M., Galan-Rodriguez, C., Madeira Da Silva, L., Saldivia, M., Karver, C. E., Rodriguez, A., Beverley, S. M., Navarro, M. and Pollastri, M. P. (2011). The susceptibility of trypanosomatid pathogens to PI3/mTOR kinase inhibitors affords a new opportunity for drug repurposing. *PLoS Neglected Tropical Diseases* **5**, e1297.
- Diro, E., Ritmeijer, K., Boelaert, M., Alves, F., Mohammed, R., Abongomera, C., Ravinetto, R., De Crop, M., Fikre, H., Adera, C., Colebunders, R., Van Loen, H., Menten, J., Lynen, L., Hailu, A. and Van Griensven, J. (2015). Use of pentamidine as secondary prophylaxis to prevent visceral leishmaniasis relapse in HIV infected patients, the first twelve months of a prospective cohort study. *PLoS Neglected Tropical Diseases* **9**, e0004087.
- Do Monte-Neto, R. L., Coelho, A. C., Raymond, F., Legare, D., Corbeil, J., Melo, M. N., Frezard, F. and Ouellette, M. (2011). Gene expression profiling and molecular characterization of antimony resistance in *Leishmania amazonensis*. *PLoS Neglected Tropical Diseases* **5**, e1167.
- Don, R., Ioset, J. R., Reguera, R. M., Calvo-Alvarez, E., Alvarez-Velilla, R. and Balana-Fouce, R. (2014). Screening strategies to identify new chemical diversity for drug development to treat kinetoplastid infections. *Parasitology* **141**, 140–146.
- Dorlo, T. P., Van Thiel, P. P., Huitema, A. D., Keizer, R. J., De Vries, H. J., Beijnen, J. H. and De Vries, P. J. (2008). Pharmacokinetics of miltefosine in Old World cutaneous leishmaniasis patients. *Antimicrobial Agents and Chemotherapy* **52**, 2855–2860.
- Dorlo, T. P., Rijal, S., Ostyn, B., De Vries, P. J., Singh, R., Bhattarai, N., Uranw, S., Dujardin, J. C., Boelaert, M., Beijnen, J. H. and Huitema, A. D. (2014). Failure of miltefosine in visceral leishmaniasis is associated with low drug exposure. *Journal of Infectious Diseases* **210**, 146–153.
- Ellenberger, T. E. and Beverley, S. M. (1989). Multiple drug resistance and conservative amplification of the H region in *Leishmania major*. *Journal of Biological Chemistry* **264**, 15094–15103.
- El-On, J. and Hamburger, A. D. (1987). Topical treatment of New and Old World cutaneous leishmaniasis in experimental animals. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 734–737.
- El-On, J., Jacobs, G. P., Witztum, E. and Greenblatt, C. L. (1984). Development of topical treatment for cutaneous leishmaniasis caused by *Leishmania major* in experimental animals. *Antimicrobial Agents and Chemotherapy* **26**, 745–751.
- El-On, J., Livshin, R., Paz, Z. E. and Weinrauch, L. (1985). Topical treatment of cutaneous leishmaniasis. *British Medical Journal* **291**, 1280–1281.
- El-On, J., Halevy, S., Grunwald, M. H. and Weinrauch, L. (1992). Topical treatment of Old World cutaneous leishmaniasis caused by *Leishmania major*: a double-blind control study. *Journal of the American Academy of Dermatology* **27**, 227–231.
- Escobar, P., Matu, S., Marques, C. and Croft, S. L. (2002). Sensitivities of *Leishmania* species to hexadecylphosphocholine (miltefosine), ET-18-OCH₃ (edelfosine) and amphotericin B. *Acta Tropica* **81**, 151–157.
- Evans, A. T., Croft, S. L., Peters, W. and Neal, R. A. (1989). Antileishmanial effects of clofazimine and other antimycobacterial agents. *Annals of Tropical Medicine and Parasitology* **83**, 447–454.
- Felizardo, T. C., Gaspar-Elsas, M. I., Lima, G. M. and Abrahamsohn, I. A. (2012). Lack of signaling by IL-4 or by IL-4/IL-13 has more attenuating effects on *Leishmania amazonensis* dorsal skin than on footpad-infected mice. *Experimental Parasitology* **130**, 48–57.
- Fernandez, M. M., Malchiodi, E. L. and Algranati, I. D. (2011). Differential effects of paromomycin on ribosomes of *Leishmania mexicana* and mammalian cells. *Antimicrobial Agents and Chemotherapy* **55**, 86–93.
- Fernandez, O. L., Diaz-Toro, Y., Ovalle, C., Valderrama, L., Muvdi, S., Rodriguez, I., Gomez, M. A. and Saravia, N. G. (2014). Miltefosine and antimonial drug susceptibility of *Leishmania Viannia* species and populations in regions of high transmission in Colombia. *PLoS Neglected Tropical Diseases* **8**, e2871.
- Fong, D., Chan, M. M., Rodriguez, R., Gately, L. J., Berman, J. D. and Grogl, M. (1994). Paromomycin resistance in *Leishmania tropica*: lack of correlation with mutation in the small subunit ribosomal RNA gene. *American Journal of Tropical Medicine and Hygiene* **51**, 758–766.
- Forestier, C. L., Spath, G. F., Prina, E., Dasari, S., Don, R., Ioset, J. R., Reguera, R. M., Calvo-Alvarez, E., Alvarez-Velilla, R. and Balana-Fouce, R. (2015). Simultaneous multi-parametric analysis of *Leishmania* and of its hosting mammal cells: a high content imaging-based method enabling sound drug discovery process. *Microbial Pathogenesis* **88**, 103–108.
- Fortin, A., Hendrickx, S., Yardley, V., Cos, P., Jansen, H. and Maes, L. (2012). Efficacy and tolerability of oleylphosphocholine (OIPC) in a laboratory model of visceral leishmaniasis. *Journal of Antimicrobial Chemotherapy* **67**, 2707–2712.
- Gadisa, E., Tsegaw, T., Abera, A., Elnaiem, D. E., Den Boer, M., Aseffa, A. and Jorge, A. (2015). Eco-epidemiology of visceral leishmaniasis in Ethiopia. *Parasites and Vectors* **8**, 381.
- Gajdos, C. and Jordan, V. C. (2002). Selective estrogen receptor modulators as a new therapeutic drug group: concept to reality in a decade. *Clinical Breast Cancer* **2**, 272–281.
- Gangwar, S., Baig, M. S., Shah, P., Biswas, S., Batra, S., Siddiqi, M. I. and Goyal, N. (2012). Identification of novel inhibitors of dipeptidylcarboxypeptidase of *Leishmania donovani* via ligand-based virtual screening and biological evaluation. *Chemical Biology & Drug Design* **79**, 149–156.
- Gazanion, E., Fernandez-Prada, C., Papadopoulou, B., Leprohon, P. and Ouellette, M. (2016). Cos-Seq for high-throughput identification of drug target and resistance mechanisms in the protozoan parasite *Leishmania*. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E3012–E3021.
- Goncalves, G. S., Fernandes, A. P., Souza, R. C., Cardoso, J. E., De Oliveira-Silva, F., Maciel, F. C., Rabello, A. and Ferreira, L. A. (2005). Activity of a paromomycin hydrophilic formulation for topical treatment of infections by *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis*. *Acta Tropica* **93**, 161–167.
- Gonzalez, U., Pinart, M., Reveiz, L. and Alvar, J. (2008). Interventions for Old World cutaneous leishmaniasis. *Cochrane Database of Systematic Reviews*, 4. Art. No.: CD005067. doi: 10.1002/14651858.CD005067.pub3.
- Gonzalez, U., Pinart, M., Rengifo-Pardo, M., Macaya, A., Alvar, J., Tweed, J. A., Gonzalez, U., Pinart, M., Reveiz, L. and Alvar, J. (2009). Interventions for American cutaneous and mucocutaneous leishmaniasis. *Cochrane Database of Systematic Reviews* **15**, CD004834.

- Gourbal, B., Sonuc, N., Bhattacharjee, H., Legare, D., Sundar, S., Ouellette, M., Rosen, B.P. and Mukhopadhyay, R. (2004). Drug uptake and modulation of drug resistance in *Leishmania* by an aquaglyceroporin. *Journal of Biological Chemistry* **279**, 31010–31017.
- Gramiccia, M., Gradoni, L. and Orsini, S. (1992). Decreased sensitivity to meglumine antimoniate (Glucontime) of *Leishmania infantum* isolated from dogs after several courses of drug treatment. *Annals of Tropical Medicine and Parasitology* **86**, 613–620.
- Grondin, K., Haimeur, A., Mukhopadhyay, R., Rosen, B.P. and Ouellette, M. (1997). Co-amplification of the gamma-glutamylcysteine synthetase gene *gsh1* and of the ABC transporter gene *pgpA* in arsenite-resistant *Leishmania tarentolae*. *EMBO Journal* **16**, 3057–3065.
- Guimond, C., Trudel, N., Brochu, C., Marquis, N., El Fadili, A., Peytavi, R., Briand, G., Richard, D., Messier, N., Papadopoulou, B., Corbeil, J., Bergeron, M.G., Legare, D. and Ouellette, M. (2003). Modulation of gene expression in *Leishmania* drug resistant mutants as determined by targeted DNA microarrays. *Nucleic Acids Research* **31**, 5886–5896.
- Gupta, S., Yardley, V., Vishwakarma, P., Shivahare, R., Sharma, B., Launay, D., Martin, D. and Puri, S.K. (2015). Nitroimidazo-oxazole compound DNDI-VL-2098: an orally effective preclinical drug candidate for the treatment of visceral leishmaniasis. *Journal of Antimicrobial Chemotherapy* **70**, 518–527.
- Hailu, A., Musa, A., Wasunna, M., Balasegaram, M., Yifru, S., Mengistu, G., Hurissa, Z., Hailu, W., Weldegebreel, T., Tesfaye, S., Makonnen, E., Khalil, E., Ahmed, O., Fadlalla, A., El-Hassan, A., Raheem, M., Mueller, M., Koummuki, Y., Rashid, J., Mbui, J., Mucee, G., Njoroge, S., Manduku, V., Musibi, A., Mutuma, G., Kirui, F., Lodenyo, H., Mutea, D., Kirigi, G., Edwards, T. et al. (2010). Geographical variation in the response of visceral leishmaniasis to paromomycin in East Africa: a multicentre, open-label, randomized trial. *PLoS Neglected Tropical Diseases* **4**, e709.
- Haimeur, A., Brochu, C., Genest, P., Papadopoulou, B. and Ouellette, M. (2000). Amplification of the ABC transporter gene PGPA and increased trypanothione levels in potassium antimonyl tartrate (SbIII) resistant *Leishmania tarentolae*. *Molecular Biochemical Parasitology* **108**, 131–135.
- Harhay, M.O., Olliaro, P.L., Costa, D.L. and Costa, C.H. (2011). Urban parasitology: visceral leishmaniasis in Brazil. *Trends in Parasitology* **27**, 403–409.
- Hendrickx, S., Eberhardt, E., Mondelaers, A., Rijal, S., Bhattarai, N.R., Dujardin, J.C., Delputte, P., Cos, P. and Maes, L. (2015). Lack of correlation between the promastigote back-transformation assay and miltefosine treatment outcome. *Journal of Antimicrobial Chemotherapy* **70**, 3023–3026.
- Hyams, K.C., Riddle, J., Trump, D.H. and Graham, J.T. (2001). Endemic infectious diseases and biological warfare during the Gulf War: a decade of analysis and final concerns. *American Journal of Tropical Medicine and Hygiene* **65**, 664–670.
- Inci, R., Ozturk, P., Mulayim, M.K., Ozyurt, K., Alatas, E.T. and Inci, M.F. (2015). Effect of the Syrian Civil War on prevalence of cutaneous leishmaniasis in Southeastern Anatolia, Turkey. *Medical Science Monitor* **21**, 2100–2104.
- Jha, S.N., Singh, N.K. and Jha, T.K. (1991). Changing response to diamidic compounds in cases of kala-azar unresponsive to antimonial. *Journal of the Association of Physicians of India* **39**, 314–316.
- Jhingran, A., Chawla, B., Saxena, S., Barrett, M.P. and Madhubala, R. (2009). Paromomycin: uptake and resistance in *Leishmania donovani*. *Molecular Biochemical Parasitology* **164**, 111–117.
- Jirmanus, L., Glesby, M.J., Guimaraes, L.H., Lago, E., Rosa, M.E., Machado, P.R. and Carvalho, E.M. (2012). Epidemiological and clinical changes in American tegumentary leishmaniasis in an area of *Leishmania (Viannia) braziliensis* transmission over a 20-year period. *American Journal of Tropical Medicine and Hygiene* **86**, 426–433.
- Kaiser, M., Maes, L., Tadoori, L.P., Spangenberg, T. and Ioset, J.R. (2015a). Repurposing of the open access malaria box for kinetoplastid diseases identifies novel active scaffolds against trypanosomatids. *Journal of Biomolecular Screening* **20**, 634–645.
- Kaiser, M., Maser, P., Tadoori, L.P., Ioset, J.R. and Brun, R. (2015b). Antiprotozoal activity profiling of approved drugs: a starting point toward drug repositioning. *PLoS ONE* **10**, e0135556.
- Karimkhani, C., Wanga, V., Coffeng, L.E., Naghavi, P., Dellavalle, R.P. and Naghavi, M. (2016). Global burden of cutaneous leishmaniasis: a cross-sectional analysis from the Global Burden of Disease Study 2013. *Lancet Infectious Diseases*, **16**, 584–91. doi: 10.1016/S1473-3099(16)00003-7.
- Kauffmann, F., Dumetz, F., Hendrickx, S., Muraille, E., Dujardin, J.C., Maes, L., Magez, S. and De Trez, C. (2016). *In vivo* characterization of two additional *Leishmania donovani* strains using the murine and hamster model. *Parasite Immunology* **38**, 290–302.
- Khare, S., Nagle, A.S., Biggart, A., Lai, Y.H., Liang, F., Davis, L.C., Barnes, S.W., Mathison, C.J., Myburgh, E., Gao, M.Y., Gillespie, J.R., Liu, X., Tan, J.L., Stinson, M., Rivera, I.C., Ballard, J., Yeh, V., Groessl, T., Federe, G., Koh, H.X., Venable, J.D., Bursulaya, B., Shapiro, M., Mishra, P.K., Spraggon, G., Brock, A., Mottram, J.C., Buckner, F.S., Rao, S.P., Wen, B.G. et al. (2016). Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. *Nature*, **537**, 229–233. doi: 10.1038/nature19339.
- Khraiwesh, M., Leed, S., Roncal, N., Johnson, J., Sciotti, R., Smith, P., Read, L., Paris, R., Hudson, T., Hickman, M. and Grogl, M. (2016). Antileishmanial activity of compounds derived from the medicines for malaria venture open access box against intracellular *Leishmania major* amastigotes. *American Journal of Tropical Medicine and Hygiene* **94**, 340–347.
- Kim, D.H., Chung, H.J., Bleys, J. and Ghohestani, R.F. (2009). Is paromomycin an effective and safe treatment against cutaneous leishmaniasis? A meta-analysis of 14 randomized controlled trials. *PLoS Neglected Tropical Diseases* **3**, e381.
- Kremsner, P.G. and Krishna, S. (2004). Antimalarial combinations. *Lancet* **364**, 285–294.
- Kreutzer, R.D., Grogl, M., Neva, F.A., Fryauff, D.J., Magill, A.J. and Aleman-Munoz, M.M. (1993). Identification and genetic comparison of leishmanial parasites causing viscerotropic and cutaneous disease in soldiers returning from Operation Desert Storm. *American Journal of Tropical Medicine and Hygiene* **49**, 357–363.
- Kuhlencord, A., Maniera, T., Eibl, H. and Unger, C. (1992). Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. *Antimicrobial Agents and Chemotherapy* **36**, 1630–1634.
- Kulshrestha, A., Singh, R., Kumar, D., Negi, N.S. and Salotra, P. (2011). Antimony-resistant clinical isolates of *Leishmania donovani* are susceptible to paromomycin and sitamaquine. *Antimicrobial Agents and Chemotherapy* **55**, 2916–2921.
- Kulshrestha, A., Sharma, V., Singh, R. and Salotra, P. (2014). Comparative transcript expression analysis of miltefosine-sensitive and miltefosine-resistant *Leishmania donovani*. *Parasitology Research* **113**, 1171–1184.
- Kumar, P., Lodge, R., Trudel, N., Ouellet, M., Ouellette, M. and Tremblay, M.J. (2010). Nelfinavir, an HIV-1 protease inhibitor, induces oxidative stress-mediated, caspase-independent apoptosis in *Leishmania* amastigotes. *PLoS Neglected Tropical Diseases* **4**, e642.
- Lachaud, L., Bourgeois, N., Plourde, M., Leprohon, P., Bastien, P. and Ouellette, M. (2009). Parasite susceptibility to amphotericin B in failures of treatment for visceral leishmaniasis in patients coinfecting with HIV type 1 and *Leishmania infantum*. *Clinical Infectious Diseases* **48**, e16–e22.
- Lang, T., Goyard, S., Lebastard, M. and Milon, G. (2005). Bioluminescent *Leishmania* expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harboring macrophages and for quantitative real-time monitoring of parasitism features in living mice. *Cellular Microbiology* **7**, 383–392.
- Lechartier, B., Rybniker, J., Zumla, A. and Cole, S.T. (2014). Tuberculosis drug discovery in the post-post-genomic era. *EMBO Molecular Medicine* **6**, 158–168.
- Legare, D., Papadopoulou, B., Roy, G., Mukhopadhyay, R., Haimeur, A., Dey, S., Grondin, K., Brochu, C., Rosen, B.P. and Ouellette, M. (1997). Efflux systems and increased trypanothione levels in arsenite-resistant *Leishmania*. *Experimental Parasitology* **87**, 275–282.
- Legare, D., Richard, D., Mukhopadhyay, R., Stierhof, Y.D., Rosen, B.P., Haimeur, A., Papadopoulou, B. and Ouellette, M. (2001). The *Leishmania* ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase. *Journal of Biological Chemistry* **276**, 26301–26307.
- Lessa, H.A., Machado, P., Lima, F., Cruz, A.A., Bacellar, O., Guerreiro, J. and Carvalho, E.M. (2001). Successful treatment of refractory mucosal leishmaniasis with pentoxifylline plus antimony. *American Journal of Tropical Medicine and Hygiene* **65**, 87–89.
- Li, C., Liu, B., Chang, J., Groessl, T., Zimmerman, M., He, Y.Q., Isbell, J., Tuntland, T., Lin, J.H., Lu, A.Y., Shearer, T.W., Smith, K.S., Diaz, D., Asher, C. and Ramirez, J. (2013). A modern *in vivo* pharmacokinetic paradigm: combining snapshot, rapid and full PK approaches to optimize and expedite early drug discovery. *Drug Discovery Today* **18**, 71–78.
- Lima, H.C., Bleyenbergh, J.A. and Titus, R.G. (1997). A simple method for quantifying *Leishmania* in tissues of infected animals. *Parasitology Today* **13**, 80–82.
- Lin, J.H. and Lu, A.Y. (1997). Role of pharmacokinetics and metabolism in drug discovery and development. *Pharmacological Reviews* **49**, 403–449.

- Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A., Saraiva, E. and Sacks, D. (1999). Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of *Leishmania donovani*. *Journal of Infectious Diseases* **180**, 564–567.
- Luque-Ortega, J. R. and Rivas, L. (2007). Miltefosine (hexadecylphosphocholine) inhibits cytochrome c oxidase in *Leishmania donovani* promastigotes. *Antimicrobial Agents and Chemotherapy* **51**, 1327–1332.
- Maarouf, M., Lawrence, F., Croft, S. L. and Robert-Gero, M. (1995). Ribosomes of *Leishmania* are a target for the aminoglycosides. *Parasitology Research* **81**, 421–425.
- Maarouf, M., De Kouchkovsky, Y., Brown, S., Petit, P. X. and Robert-Gero, M. (1997). *In vivo* interference of paromomycin with mitochondrial activity of *Leishmania*. *Experimental Cell Research* **232**, 339–348.
- Machado, P. R., Lessa, H., Lessa, M., Guimaraes, L. H., Bang, H., Ho, J. L. and Carvalho, E. M. (2007). Oral pentoxifylline combined with pentavalent antimony: a randomized trial for mucosal leishmaniasis. *Clinical Infectious Diseases* **44**, 788–793.
- Machado, P. R., Ampuero, J., Guimaraes, L. H., Villasboas, L., Rocha, A. T., Schriefer, A., Sousa, R. S., Talhari, A., Penna, G. and Carvalho, E. M. (2010). Miltefosine in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil: a randomized and controlled trial. *PLoS Neglected Tropical Diseases* **4**, e912.
- Mahajan, R., Das, P., Isaakidis, P., Sunyoto, T., Sagili, K. D., Lima, M. A., Mitra, G., Kumar, D., Pandey, K., Van Geertruyden, J. P., Boelaert, M. and Burza, S. (2015). Combination treatment for visceral leishmaniasis patients coinfecting with human immunodeficiency virus in India. *Clinical Infectious Diseases* **61**, 1255–1262.
- Manandhar, K. D., Yadav, T. P., Prajapati, V. K., Kumar, S., Rai, M., Dube, A., Srivastava, O. N. and Sundar, S. (2008). Antileishmanial activity of nano-amphotericin B deoxycholate. *Journal of Antimicrobial Chemotherapy* **62**, 376–380.
- Manjunatha, U. H., Smith, P. W., Lechartier, B., Rybniker, J., Zumla, A. and Cole, S. T. (2015). Perspective: challenges and opportunities in TB drug discovery from phenotypic screening. *Bioorganic and Medicinal Chemistry* **23**, 5087–5097.
- Manzano, J. I., Garcia-Hernandez, R., Castanys, S. and Gamarro, F. (2013). A new ABC half-transporter in *Leishmania major* is involved in resistance to antimony. *Antimicrobial Agents and Chemotherapy* **57**, 3719–3730.
- Marsden, P. D. (1985). Pentavalent antimonials: old drugs for new diseases. *Revista da Sociedade Brasileira de Medicina Tropical* **18**, 187–198.
- Mbongo, N., Loiseau, P. M., Billion, M. A. and Robert-Gero, M. (1998). Mechanism of amphotericin B resistance in *Leishmania donovani* promastigotes. *Antimicrobial Agents and Chemotherapy* **42**, 352–357.
- Melaku, Y., Collin, S. M., Keus, K., Gatluak, F., Ritmeijer, K. and Davidson, R. N. (2007). Treatment of kala-azar in southern Sudan using a 17-day regimen of sodium stibogluconate combined with paromomycin: a retrospective comparison with 30-day sodium stibogluconate monotherapy. *American Journal of Tropical Medicine and Hygiene* **77**, 89–94.
- Michel, G., Ferrua, B., Lang, T., Maddugoda, M. P., Munro, P., Pomares, C., Lemichez, E. and Marty, P. (2011). Luciferase-expressing *Leishmania infantum* allows the monitoring of amastigote population size, *in vivo*, *ex vivo* and *in vitro*. *PLoS Neglected Tropical Diseases* **5**, e1323.
- Miguel, D. C., Yokoyama-Yasunaka, J. K., Andreoli, W. K., Mortara, R. A. and Uliana, S. R. (2007). Tamoxifen is effective against *Leishmania* and induces a rapid alkalization of parasitophorous vacuoles harbouring *Leishmania (Leishmania) amazonensis* amastigotes. *Journal of Antimicrobial Chemotherapy* **60**, 526–534.
- Miguel, D. C., Zauli-Nascimento, R. C., Yokoyama-Yasunaka, J. K., Katz, S., Barbieri, C. L. and Uliana, S. R. (2009). Tamoxifen as a potential antileishmanial agent: efficacy in the treatment of *Leishmania braziliensis* and *Leishmania chagasi* infections. *Journal of Antimicrobial Chemotherapy* **63**, 365–368.
- Miguel, D. C., Zauli-Nascimento, R. C., Yokoyama-Yasunaka, J. K., Pereira, L. I., Jeronimo, S. M., Ribeiro-Dias, F., Dorta, M. L. and Uliana, S. R. (2011). Clinical isolates of New World *Leishmania* from cutaneous and visceral leishmaniasis patients are uniformly sensitive to tamoxifen. *International Journal of Antimicrobial Agents* **38**, 93–94.
- Mina, J. G., Mosely, J. A., Ali, H. Z., Shams-Eldin, H., Schwarz, R. T., Steel, P. G. and Denny, P. W. (2010). A plate-based assay system for analyses and screening of the *Leishmania major* inositol phosphorylceramide synthase. *International Journal of Biochemistry and Cell Biology* **42**, 1553–1561.
- Mitchison, D. and Davies, G. (2012). The chemotherapy of tuberculosis: past, present and future. *International Journal of Tuberculosis and Lung Disease* **16**, 724–732.
- Mohebbi, M., Fotouhi, A., Hooshmand, B., Zarei, Z., Akhondi, B., Rahnama, A., Razaghian, A. R., Kabir, M. J. and Nadim, A. (2007). Comparison of miltefosine and meglumine antimoniate for the treatment of zoonotic cutaneous leishmaniasis (ZCL) by a randomized clinical trial in Iran. *Acta Tropica* **103**, 33–40.
- Molina, R., Gradoni, L. and Alvar, J. (2003). HIV and the transmission of *Leishmania*. *Annals of Tropical Medicine and Parasitology* **97** (Suppl. 1), 29–45.
- Mondelaers, A., Sanchez-Canete, M. P., Hendrickx, S., Eberhardt, E., Garcia-Hernandez, R., Lachaud, L., Cotton, J., Sanders, M., Cuyppers, B., Imamura, H., Dujardin, J. C., Delputte, P., Cos, P., Caljon, G., Gamarro, F., Castanys, S. and Maes, L. (2016). Genomic and molecular characterization of miltefosine resistance in *Leishmania infantum* strains with either natural or acquired resistance through experimental selection of intracellular amastigotes. *PLoS ONE* **11**, e0154101.
- Monte-Neto, R., Laffitte, M. C., Leprohon, P., Reis, P., Frezard, F. and Ouellette, M. (2015). Intrachromosomal amplification, locus deletion and point mutation in the aquaglyceroporin AQP1 gene in antimony resistant *Leishmania (Viannia) guyanensis*. *PLoS Neglected Tropical Diseases* **9**, e0003476.
- Moreira, N., Vitoriano-Souza, J., Roatt, B. M., Vieira, P. M., Coura-Vital, W., Cardoso, J. M., Rezende, M. T., Ker, H. G., Giunchetti, R. C., Carneiro, C. M. and Reis, A. B. (2016). Clinical, hematological and biochemical alterations in hamster (*Mesocricetus auratus*) experimentally infected with *Leishmania infantum* through different routes of inoculation. *Parasites and Vectors* **9**, 181.
- Mukherjee, A., Padmanabhan, P. K., Singh, S., Roy, G., Girard, I., Chatterjee, M., Ouellette, M. and Madhubala, R. (2007). Role of ABC transporter MRPA, gamma-glutamylcysteine synthetase and ornithine decarboxylase in natural antimony-resistant isolates of *Leishmania donovani*. *Journal of Antimicrobial Chemotherapy* **59**, 204–211.
- Mukhopadhyay, R., Dey, S., Xu, N., Gage, D., Lightbody, J., Ouellette, M. and Rosen, B. P. (1996). Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 10383–10387.
- Murray, C. J., B. R., Foreman, K. J., Abbasoglu Ozgoren, A., Abd-Allah, F., Abera, S. F., Aboyans, V., Abraham, J. P., Abubakar, I., Abu-Raddad, L. J., Abu-Rmeileh, N. M., Achoki, T., Ackerman, I. N., Ademi, Z., Adou, A. K., Adsuar, J. C., Afshin, A., Agardh, E. E., Alam, S. S., Alasfoor, D., Albittar, M. I., Alegretti, M. A., Alemu, Z. A., Alfonso-Cristancho, R., Alhabib, S., Ali, R., Alla, F., Allebeck, P., Almazroa, M. A. and Alsharif, U. *et al.* (2016). Leishmaniasis in high-burden countries: an epidemiological update based on data reported in 2014. *Weekly Epidemiological Record* **91**, 287–296.
- Musa, A., Khalil, E., Hailu, A., Olobo, J., Balasegaram, M., Omollo, R., Edwards, T., Rashid, J., Mbuyi, J., Musa, B., Abuzaid, A. A., Ahmed, O., Fadlalla, A., El-Hassan, A., Mueller, M., Mucee, G., Njoroge, S., Manduku, V., Mutuma, G., Apadet, Z. A., Lodenyo, H., Mutea, D., Kirigi, G., Yifru, S., Mengistu, G., Hurissa, Z., Hailu, W., Weldegebreal, T., Tafes, H., Mekonnen, Y. *et al.* (2012). Sodium stibogluconate (SSG) & paromomycin combination compared to SSG for visceral leishmaniasis in East Africa: a randomised controlled trial. *PLoS Neglected Tropical Diseases* **6**, e1674.
- Musa, A. M., Younis, B., Fadlalla, A., Royce, C., Balasegaram, M., Wasunna, M., Hailu, A., Edwards, T., Omollo, R., Mudawi, M., Kokwaro, G., El-Hassan, A. and Khalil, E. (2010). Paromomycin for the treatment of visceral leishmaniasis in Sudan: a randomized, open-label, dose-finding study. *PLoS Neglected Tropical Diseases* **4**, e855.
- Nabors, G. S., Afonso, L. C., Farrell, J. P. and Scott, P. (1995). Switch from a type 2 to a type 1T helper cell response and cure of established *Leishmania major* infection in mice is induced by combined therapy with interleukin 12 and Pentostam. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3142–3146.
- Navin, T. R., Arana, B. A., Arana, F. E., Berman, J. D. and Chajon, J. F. (1992). Placebo-controlled clinical trial of sodium stibogluconate (Pentostam) versus ketoconazole for treating cutaneous leishmaniasis in Guatemala. *Journal of Infectious Diseases* **165**, 528–534.
- Neal, R. A., Gonzalez, U., Pinart, M., Rengifo-Pardo, M., Macaya, A., Alvar, J., Tweed, J. A., Gonzalez, U., Pinart, M., Reveiz, L. and Alvar, J. (1968). The effect of antibiotics of the neomycin group on experimental cutaneous leishmaniasis. *Annals of Tropical Medicine and Parasitology* **62**, 54–62.
- Neal, R. A., Allen, S., Mccoy, N., Olliaro, P. and Croft, S. L. (1995). The sensitivity of *Leishmania* species to aminosidine. *Journal of Antimicrobial Chemotherapy* **35**, 577–584.
- Neves, L. O., Talhari, A. C., Gadelha, E. P., Silva, R. M., Jr., Guerra, J. A., Ferreira, L. C. and Talhari, S. (2011). A randomized clinical trial comparing meglumine antimoniate, pentamidine and amphotericin B for the treatment of cutaneous leishmaniasis by *Leishmania guyanensis*. *Anais Brasileiros de Dermatologia* **86**, 1092–1101.

- Nicolas, L., Prina, E., Lang, T. and Milon, G. (2002). Real-time PCR for detection and quantitation of leishmania in mouse tissues. *Journal of Clinical Microbiology* **40**, 1666–1669.
- Nuhs, A., Schafer, C., Zander, D., Trube, L., Tejera Nevado, P., Schmidt, S., Arevalo, J., Adaui, V., Maes, L., Dujardin, J. C. and Clos, J. (2014). A novel marker, ARM58, confers antimony resistance to *Leishmania* spp. *International Journal of Parasitology: Drugs and Drug Resistance* **4**, 37–47.
- Obonaga, R., Fernandez, O. L., Valderrama, L., Rubiano, L. C., Castro Mdel, M., Barrera, M. C., Gomez, M. A. and Gore Saravia, N. (2014). Treatment failure and miltefosine susceptibility in dermal leishmaniasis caused by *Leishmania* subgenus *Viannia* species. *Antimicrobial Agents and Chemotherapy* **58**, 144–152.
- Olliaro, P. L., Guerin, P. J., Gerstl, S., Haaskjold, A. A., Rottingen, J. A. and Sundar, S. (2005). Treatment options for visceral leishmaniasis: a systematic review of clinical studies done in India, 1980–2004. *Lancet Infectious Diseases* **5**, 763–774.
- Omollo, R., Alexander, N., Edwards, T., Khalil, E. A., Younis, B. M., Abuzaid, A. A., Wasunna, M., Njoroge, N., Kinoti, D., Kirigi, G., Dorlo, T. P., Ellis, S., Balasegaram, M. and Musa, A. M. (2011). Safety and efficacy of miltefosine alone and in combination with sodium stibogluconate and liposomal amphotericin B for the treatment of primary visceral leishmaniasis in East Africa: study protocol for a randomized controlled trial. *Trials* **12**, 166.
- Pandey, R. K., Sharma, D., Bhatt, T. K., Sundar, S. and Prajapati, V. K. (2015). Developing imidazole analogues as potential inhibitor for *Leishmania donovani* trypanothione reductase: virtual screening, molecular docking, dynamics and ADMET approach. *Journal of Biomolecular Structure and Dynamics* **33**, 2541–2553.
- Papadopoulou, B., Roy, G., Dey, S., Rosen, B. P. and Ouellette, M. (1994). Contribution of the *Leishmania* P-glycoprotein-related gene *ltpgpA* to oxanyon resistance. *Journal of Biological Chemistry* **269**, 11980–11986.
- Patterson, S., Wyllie, S., Norval, S., Stojanovski, L., Simeons, F. R., Auer, J. L., Osuna-Cabello, M., Read, K. D. and Fairlamb, A. H. (2016). The anti-tubercular drug delamanid as a potential oral treatment for visceral leishmaniasis. *Elife* **5**, e09744.
- Pena, I., Pilar Manzano, M., Cantizani, J., Kessler, A., Alonso-Padilla, J., Bardera, A. I., Alvarez, E., Colmenarejo, G., Cotillo, I., Roquero, I., De Dios-Anton, F., Barroso, V., Rodriguez, A., Gray, D. W., Navarro, M., Kumar, V., Sherstnev, A., Drewry, D. H., Brown, J. R., Fiandor, J. M. and Julio Martin, J. (2015). New compound sets identified from high throughput phenotypic screening against three kinetoplast parasites: an open resource. *Scientific Reports* **5**, 8771.
- Perea, A., Manzano, J. I., Castanys, S. and Gamarro, F. (2016). The LABC2 transporter from the protozoan parasite *Leishmania* is involved in antimony resistance. *Antimicrobial Agents and Chemotherapy* **60**, 3489–3496.
- Perez-Victoria, F. J., Gamarro, F., Ouellette, M. and Castanys, S. (2003). Functional cloning of the miltefosine transporter. A novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. *Journal of Biological Chemistry* **278**, 49965–49971.
- Perez-Victoria, F. J., Sanchez-Canete, M. P., Castanys, S. and Gamarro, F. (2006a). Phospholipid translocation and miltefosine potency require both *L. donovani* miltefosine transporter and the new protein LdRos3 in *Leishmania* parasites. *Journal of Biological Chemistry* **281**, 23766–23775.
- Perez-Victoria, F. J., Sanchez-Canete, M. P., Seifert, K., Croft, S. L., Sundar, S., Castanys, S. and Gamarro, F. (2006b). Mechanisms of experimental resistance of *Leishmania* to miltefosine: implications for clinical use. *Drug Resistance Updates* **9**, 26–39.
- Perez-Victoria, J. M., Perez-Victoria, F. J., Parodi-Talice, A., Jimenez, I. A., Ravelo, A. G., Castanys, S. and Gamarro, F. (2001). Alkyl-lysophospholipid resistance in multidrug-resistant *Leishmania tropica* and chemosensitization by a novel P-glycoprotein-like transporter modulator. *Antimicrobial Agents and Chemotherapy* **45**, 2468–2474.
- Perry, M., Wyllie, S., Prajapati, V., Menten, J., Raab, A., Feldmann, J., Chakraborti, D., Sundar, S., Boelaert, M., Picado, A. and Fairlamb, A. (2015). Arsenic, antimony, and *Leishmania*: has arsenic contamination of drinking water in India led to treatment-resistant kala-azar? *Lancet* **385** (Suppl. 1), S80.
- Prajapati, V. K., Awasthi, K., Gautam, S., Yadav, T. P., Rai, M., Srivastava, O. N. and Sundar, S. (2011). Targeted killing of *Leishmania donovani* in vivo and in vitro with amphotericin B attached to functionalized carbon nanotubes. *Journal of Antimicrobial Chemotherapy* **66**, 874–879.
- Prajapati, V. K., Mehrotra, S., Gautam, S., Rai, M. and Sundar, S. (2012). In vitro antileishmanial drug susceptibility of clinical isolates from patients with Indian visceral leishmaniasis – status of newly introduced drugs. *American Journal of Tropical Medicine and Hygiene* **87**, 655–657.
- Prajapati, V. K., Sharma, S., Rai, M., Ostyn, B., Salotra, P., Vanaerschot, M., Dujardin, J. C. and Sundar, S. (2013). In vitro susceptibility of *Leishmania donovani* to miltefosine in Indian visceral leishmaniasis. *American Journal of Tropical Medicine and Hygiene* **89**, 750–754.
- Prata, A. (1963). Treatment of kala-azar with amphotericin B. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **57**, 266–268.
- Purkait, B., Kumar, A., Nandi, N., Sardar, A. H., Das, S., Kumar, S., Pandey, K., Ravidas, V., Kumar, M., De, T., Singh, D. and Das, P. (2012). Mechanism of amphotericin B resistance in clinical isolates of *Leishmania donovani*. *Antimicrobial Agents and Chemotherapy* **56**, 1031–1041.
- Purkait, B., Singh, R., Wasnik, K., Das, S., Kumar, A., Paine, M., Dikhit, M., Singh, D., Sardar, A. H., Ghosh, A. K. and Das, P. (2015). Up-regulation of silent information regulator 2 (Sir2) is associated with amphotericin B resistance in clinical isolates of *Leishmania donovani*. *Journal of Antimicrobial Chemotherapy* **70**, 1343–1356.
- Rackham, M. D., Yu, Z., Brannigan, J. A., Heal, W. P., Paape, D., Barker, K. V., Wilkinson, A. J., Smith, D. F., Leatherbarrow, R. J. and Tate, E. W. (2015). Discovery of high affinity inhibitors of *Leishmania donovani* N-myristoyltransferase. *Medchemcomm* **6**, 1761–1766.
- Rai, K., Cuypers, B., Bhattarai, N. R., Uranw, S., Berg, M., Ostyn, B., Dujardin, J. C., Rijal, S. and Vanaerschot, M. (2013). Relapse after treatment with miltefosine for visceral leishmaniasis is associated with increased infectivity of the infecting *Leishmania donovani* strain. *MBio* **4**, e00611–e00613.
- Rakotomanga, M., Blanc, S., Gaudin, K., Chaminade, P. and Loiseau, P. M. (2007). Miltefosine affects lipid metabolism in *Leishmania donovani* promastigotes. *Antimicrobial Agents and Chemotherapy* **51**, 1425–1430.
- Reguera, R. M., Calvo-Alvarez, E., Alvarez-Velilla, R. and Balana-Fouce, R. (2014). Target-based vs. phenotypic screenings in *Leishmania* drug discovery: a marriage of convenience or a dialogue of the deaf? *International Journal of Parasitology: Drugs and Drug Resistance* **4**, 355–357.
- Reimao, J. Q., Colombo, F. A., Pereira-Chioccola, V. L. and Tempone, A. G. (2011). In vitro and experimental therapeutic studies of the calcium channel blocker bepridil: detection of viable *Leishmania (L.) chagasi* by real-time PCR. *Experimental Parasitology* **128**, 111–115.
- Reimao, J. Q., Trinconi, C. T., Yokoyama-Yasunaka, J. K., Miguel, D. C., Kalil, S. P. and Uliana, S. R. (2013). Parasite burden in *Leishmania (Leishmania) amazonensis*-infected mice: validation of luciferase as a quantitative tool. *Journal of Microbiological Methods* **93**, 95–101.
- Reimao, J. Q., Oliveira, J. C., Trinconi, C. T., Cotrim, P. C., Coelho, A. C. and Uliana, S. R. (2015). Generation of luciferase-expressing *Leishmania infantum chagasi* and assessment of miltefosine efficacy in infected hamsters through bioimaging. *PLoS Neglected Tropical Diseases* **9**, e0003556.
- Ribeiro-Romao, R. P., Moreira, O. C., Osorio, E. Y., Cysne-Finkelstein, L., Gomes-Silva, A., Valverde, J. G., Pirmez, C., Da-Cruz, A. M. and Pinto, E. F. (2014). Comparative evaluation of lesion development, tissue damage, and cytokine expression in golden hamsters (*Mesocricetus auratus*) infected by inocula with different *Leishmania (Viannia) braziliensis* concentrations. *Infection and Immunity* **82**, 5203–5213.
- Richardson, J. L., Nett, I. R., Jones, D. C., Abdille, M. H., Gilbert, I. H. and Fairlamb, A. H. (2009). Improved tricyclic inhibitors of trypanothione reductase by screening and chemical synthesis. *ChemMedChem* **4**, 1333–1340.
- Rijal, S., Ostyn, B., Uranw, S., Rai, K., Bhattarai, N. R., Dorlo, T. P., Beijnen, J. H., Vanaerschot, M., Decuypere, S., Dhakal, S. S., Das, M. L., Karki, P., Singh, R., Boelaert, M. and Dujardin, J. C. (2013). Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or non-compliance. *Clinical Infectious Diseases* **56**, 1530–1538.
- Riley, R. J., Kenna, J. G., Li, C., Liu, B., Chang, J., Groessl, T., Zimmerman, M., He, Y. Q., Isbell, J., Tuntland, T., Shearer, T. W., Smith, K. S., Diaz, D., Asher, C., Ramirez, J., Lin, J. H. and Lu, A. Y. (2004). Cellular models for ADMET predictions and evaluation of drug-drug interactions. *Current Opinion in Drug Discovery and Development* **7**, 86–99.
- Ritmeijer, K., Veeken, H., Melaku, Y., Leal, G., Amsalu, R., Seaman, J. and Davidson, R. N. (2001). Ethiopian visceral leishmaniasis: generic and proprietary sodium stibogluconate are equivalent; HIV co-infected patients have a poor outcome. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **95**, 668–672.

- Roberts, W. L. and Rainey, P. M. (1993). Antileishmanial activity of sodium stibogluconate fractions. *Antimicrobial Agents and Chemotherapy* **37**, 1842–1846.
- Sacks, D. and Kamhawi, S. (2001). Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annual Review of Microbiology* **55**, 453–483.
- Sadeghian, G. and Nilfroushzadeh, M. A. (2006). Effect of combination therapy with systemic glucantime and pentoxifylline in the treatment of cutaneous leishmaniasis. *International Journal of Dermatology* **45**, 819–821.
- Sampaio, S. A., Godoy, J. T., Paiva, L., Dillon, N. L. and Da, L. C. (1960). The treatment of American (mucocutaneous) leishmaniasis with amphotericin B. *Archives of Dermatology* **82**, 627–635.
- Sanchez-Brunete, J. A., Dea, M. A., Rama, S., Bolas, F., Alunda, J. M., Raposo, R., Mendez, M. T., Torrado-Santiago, S. and Torrado, J. J. (2004). Treatment of experimental visceral leishmaniasis with amphotericin B in stable albumin microspheres. *Antimicrobial Agents and Chemotherapy* **48**, 3246–3252.
- Sanchez-Canete, M. P., Carvalho, L., Perez-Victoria, F. J., Gamarro, F. and Castanys, S. (2009). Low plasma membrane expression of the miltefosine transport complex renders *Leishmania braziliensis* refractory to the drug. *Antimicrobial Agents and Chemotherapy* **53**, 1305–1313.
- Sanderson, L., Yardley, V. and Croft, S. L. (2014). Activity of anti-cancer protein kinase inhibitors against *Leishmania* spp. *Journal of Antimicrobial Chemotherapy* **69**, 1888–1891.
- Santa-Rita, R. M., Henriques-Pons, A., Barbosa, H. S. and De Castro, S. L. (2004). Effect of the lysophospholipid analogues edelfosine, ilmofosine and miltefosine against *Leishmania amazonensis*. *Journal of Antimicrobial Chemotherapy* **54**, 704–710.
- Santos, D. M., Petersen, A. L., Celes, F. S., Borges, V. M., Veras, P. S. and De Oliveira, C. I. (2014). Chemotherapeutic potential of 17-AAG against cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis*. *PLoS Neglected Tropical Diseases* **8**, e3275.
- Santos, L. O., Vitorio, B. S., Branquinha, M. H., Pedrosa E Silva, C. M., Santos, A. L. and D'Avila-Levy, C. M. (2013). Nelfinavir is effective in inhibiting the multiplication and aspartic peptidase activity of *Leishmania* species, including strains obtained from HIV-positive patients. *Journal of Antimicrobial Chemotherapy* **68**, 348–353.
- Seaman, J., Pryce, D., Sondorp, H. E., Moody, A., Bryceson, A. D. and Davidson, R. N. (1993). Epidemic visceral leishmaniasis in Sudan: a randomized trial of aminosidine plus sodium stibogluconate versus sodium stibogluconate alone. *Journal of Infectious Diseases* **168**, 715–720.
- Seifert, K., Matu, S., Javier Perez-Victoria, F., Castanys, S., Gamarro, F. and Croft, S. L. (2003). Characterisation of *Leishmania donovani* promastigotes resistant to hexadecylphosphocholine (miltefosine). *International Journal of Antimicrobial Agents* **22**, 380–387.
- Seifert, K., Perez-Victoria, F. J., Stettler, M., Sanchez-Canete, M. P., Castanys, S., Gamarro, F. and Croft, S. L. (2007). Inactivation of the miltefosine transporter, LdMT, causes miltefosine resistance that is conferred to the amastigote stage of *Leishmania donovani* and persists *in vivo*. *International Journal of Antimicrobial Agents* **30**, 229–235.
- Sereno, D. and Lemesre, J. L. (1997). *In vitro* life cycle of pentamidine-resistant amastigotes: stability of the chemoresistant phenotypes is dependent on the level of resistance induced. *Antimicrobial Agents and Chemotherapy* **41**, 1898–1903.
- Shaked-Mishan, P., Ulrich, N., Ephros, M. and Zilberstein, D. (2001). Novel Intracellular SbV reducing activity correlates with antimony susceptibility in *Leishmania donovani*. *Journal of Biological Chemistry* **276**, 3971–3976.
- Shalev, M., Kondo, J., Kopelyanskiy, D., Jaffe, C. L., Adir, N., and Baasov, T. (2013). Identification of the molecular attributes required for aminoglycoside activity against *Leishmania*. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 13333–13338.
- Sharara, S. L. and Kanj, K. S. (2014). War and infectious diseases: challenges of the Syrian civil war. *PLoS Pathogens* **10**, e1004438.
- Shaw, C. D., Lonchamp, J., Downing, T., Imamura, H., Freeman, T. M., Cotton, J. A., Sanders, M., Blackburn, G., Dujardin, J. C., Rijal, S., Khanal, B., Illingworth, C. J., Coombs, G. H. and Carter, K. C. (2016). *In vitro* selection of miltefosine resistance in promastigotes of *Leishmania donovani* from Nepal: genomic and metabolomic characterization. *Molecular Microbiology* **99**, 1134–1148.
- Shearer, T. W., Smith, K. S., Diaz, D., Asher, C., Ramirez, J., Riley, R. J., Kenna, J. G., Li, C., Liu, B., Chang, J., Groessl, T., Zimmerman, M., He, Y. Q., Isbell, J. and Tuntland, T. (2005). The role of *in vitro* ADME assays in antimalarial drug discovery and development. *Combinatorial Chemistry and High Throughput Screening* **8**, 89–98.
- Singh, A. K., Papadopoulou, B. and Ouellette, M. (2001). Gene amplification in amphotericin B-resistant *Leishmania tarentolae*. *Experimental Parasitology* **99**, 141–147.
- Siqueira-Neto, J. L., Moon, S., Jang, J., Yang, G., Lee, C., Moon, H. K., Chatelain, E., Genovesio, A., Cechetto, J. and Freitas-Junior, L. H. (2012). An image-based high-content screening assay for compounds targeting intracellular *Leishmania donovani* amastigotes in human macrophages. *PLoS Neglected Tropical Diseases* **6**, e1671.
- Soto, J. and Berman, J. (2006). Treatment of New World cutaneous leishmaniasis with miltefosine. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **100** (Suppl. 1), S34–S40.
- Soto, J., Fuya, P., Herrera, R. and Berman, J. (1998). Topical paromomycin/methylbenzethonium chloride plus parenteral meglumine antimonate as treatment for American cutaneous leishmaniasis: controlled study. *Clinical Infectious Diseases* **26**, 56–58.
- Spath, G. F. and Beverley, S. M. (2001). A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. *Experimental Parasitology* **99**, 97–103.
- Srivastava, A., Sweat, J. M., Azizan, A., Vesely, B. and Kyle, D. E. (2013). Real-time PCR to quantify *Leishmania donovani* in hamsters. *Journal of Parasitology* **99**, 145–150.
- Sundar, S. (2001). Drug resistance in Indian visceral leishmaniasis. *Tropical Medicine and International Health* **6**, 849–854.
- Sundar, S., Jha, T. K., Thakur, C. P., Engel, J., Sindermann, H., Fischer, C., Junge, K., Bryceson, A. and Berman, J. (2002). Oral miltefosine for Indian visceral leishmaniasis. *New England Journal of Medicine* **347**, 1739–1746.
- Sundar, S., Jha, T. K., Thakur, C. P., Sinha, P. K. and Bhattacharya, S. K. (2007). Injectable paromomycin for Visceral leishmaniasis in India. *New England Journal of Medicine* **356**, 2571–2581.
- Sundar, S., Rai, M., Chakravarty, J., Agarwal, D., Agrawal, N., Vaillant, M., Olliaro, P. and Murray, H. W. (2008). New treatment approach in Indian visceral leishmaniasis: single-dose liposomal amphotericin B followed by short-course oral miltefosine. *Clinical Infectious Diseases* **47**, 1000–1006.
- Sundar, S., Sinha, P. K., Rai, M., Verma, D. K., Nawin, K., Alam, S., Chakravarty, J., Vaillant, M., Verma, N., Pandey, K., Kumari, P., Lal, C. S., Arora, R., Sharma, B., Ellis, S., Strub-Wourgaft, N., Balasegaram, M., Olliaro, P., Das, P. and Modabber, F. (2011). Comparison of short-course multidrug treatment with standard therapy for visceral leishmaniasis in India: an open-label, non-inferiority, randomised controlled trial. *Lancet* **377**, 477–486.
- Sundar, S., Singh, A., Rai, M., Prajapati, V. K., Singh, A. K., Ostyn, B., Boelaert, M., Dujardin, J. C. and Chakravarty, J. (2012). Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clinical Infectious Diseases* **55**, 543–550.
- Szoka, F. C., Jr., Milholland, D. and Barza, M. (1987). Effect of lipid composition and liposome size on toxicity and *in vitro* fungicidal activity of liposome-intercalated amphotericin B. *Antimicrobial Agents and Chemotherapy* **31**, 421–429.
- Thakur, C. P. (1984). Epidemiological, clinical and therapeutic features of Bihar kala-azar (including post kala-azar dermal leishmaniasis). *Transactions of the Royal Society of Tropical Medicine and Hygiene* **78**, 391–398.
- Thakur, C. P., Kumar, M., Singh, S. K., Sharma, D., Prasad, U. S., Singh, R. S., Dhawan, P. S. and Achari, V. (1984). Comparison of regimens of treatment with sodium stibogluconate in kala-azar. *British Medical Journal (Clinical Research Ed.)* **288**, 895–897.
- Thakur, C. P., Olliaro, P., Gothoskar, S., Bhowmick, S., Choudhury, B. K., Prasad, S., Kumar, M. and Verma, B. B. (1992). Treatment of visceral leishmaniasis (kala-azar) with aminosidine (= paromomycin)-antimonial combinations, a pilot study in Bihar, India. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86**, 615–616.
- Valdivieso, E., Rangel, A., Moreno, J., Saugar, J. M., Canavate, C., Alvar, J. and Dagger, F. (2010). Effects of HIV aspartyl-proteinase inhibitors on *Leishmania* sp. *Experimental Parasitology* **126**, 557–563.
- Vandamme, A. M., Van Laethem, K. and De Clercq, E. (1999). Managing resistance to anti-HIV drugs: an important consideration for effective disease management. *Drugs* **57**, 337–361.
- Van Der Meide, W. F., Sabajo, L. O., Jensema, A. J., Peekel, I., Faber, W. R., Schallig, H. D. and Fat, R. F. (2009). Evaluation of treatment with pentamidine for cutaneous leishmaniasis in Suriname. *International Journal of Dermatology* **48**, 52–58.
- Van Griensven, J., Balasegaram, M., Meheus, F., Alvar, J., Lynen, L. and Boelaert, M. (2010). Combination therapy for visceral leishmaniasis. *Lancet Infectious Diseases* **10**, 184–194.
- Van Griensven, J., Diro, E., Lopez-Velez, R., Boelaert, M., Lynen, L., Zijlstra, E., Dujardin, J. C. and Hailu, A. (2013). HIV-1 protease inhibitors for treatment of visceral leishmaniasis in HIV-co-infected individuals. *Lancet Infectious Diseases* **13**, 251–259.

- Vianna, G.** (1914). Sobre o tratamento de leishmaniose tegumentar. *Anais Paulista de Medicina e Cirurgia* **2**, 167–169.
- WHO** (1984). The leishmaniasis. Report of a WHO Expert Committee. *World Health Organization Technical Report Series* **701**, 1–140.
- WHO** (2010). Control of the leishmaniasis: report of a meeting of the World Health Organization Expert Committee on the Control of Leishmaniasis. Geneva, 22–26 March 2010. *World Health Organization Technical Report Series* **949**, 1–186.
- WHO** (2012). Research priorities for Chagas disease, human African trypanosomiasis and leishmaniasis. *World Health Organization Technical Report Series* **975**, 1–100.
- Wyllie, S., Patterson, S., Stojanovski, L., Simeons, F. R., Norval, S., Kime, R., Read, K. D. and Fairlamb, A. H.** (2012). The anti-trypanosome drug fexinidazole shows potential for treating visceral leishmaniasis. *Science Translational Medicine* **4**, 119re111.
- Yardley, V. and Croft, S. L.** (1997). Activity of liposomal amphotericin B against experimental cutaneous leishmaniasis. *Antimicrobial Agents and Chemotherapy* **41**, 752–756.
- Yardley, V., Croft, S. L., De Doncker, S., Dujardin, J. C., Koirala, S., Rijal, S., Miranda, C., Llanos-Cuentas, A. and Chappuis, F.** (2005). The sensitivity of clinical isolates of *Leishmania* from Peru and Nepal to miltefosine. *American Journal of Tropical Medicine and Hygiene* **73**, 272–275.
- Zhou, Y., Messier, N., Ouellette, M., Rosen, B. P. and Mukhopadhyay, R.** (2004). *Leishmania major* LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug pentostam. *Journal of Biological Chemistry* **279**, 37445–37451.