Expert Opinion on Drug Discovery

ISSN: 1746-0441 (Print) 1746-045X (Online) Journal homepage: https://www.tandfonline.com/loi/iedc20

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To cite this article: Juliana Q. Reimão, Débora P. Pita Pedro & Adriano C. Coelho (2020): The preclinical discovery and development of oral miltefosine for the treatment of visceral leishmaniasis: a case history, Expert Opinion on Drug Discovery, DOI: 10.1080/17460441.2020.1743674

To link to this article: https://doi.org/10.1080/17460441.2020.1743674

Published online: 23 Mar 2020.

EXPERT OPINION ON DRUG DISCOVERY
https://doi.org/10.1080/17460441.2020.1743674

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The preclinical discovery and development of oral miltefosine for the treatment of visceral leishmaniasis: a case history

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ABSTRACT

Introduction: Visceral leishmaniasis (VL) is a vector-borne disease caused by *Leishmania donovani* or *Leishmania infantum*. Closely related to poverty, VL is fatal and represents one of the main burdens on public health in developing countries. Treatment of VL relies exclusively on chemotherapy, a strategy still experiencing numerous limitations. Miltefosine (MF) has been used in the chemotherapy of VL in some endemic areas, and has been expanded to other regions, being considered crucial in eradication programs.

Areas covered: This article reviews the most relevant preclinical and clinical aspects of MF, its mechanism of action and resistance to *Leishmania* parasites, as well as its limitations. The authors also give their perspectives on the treatment of VL.

Expert opinion: The discovery of MF represented an enormous advance in the chemotherapy of VL, since it was the first oral drug for this neglected disease. Beyond selection of resistant parasites due to drug pressure, several other factors can lead to treatment failure such as, for example, factors intrinsic to the host, parasite and the drug itself. Although its efficacy as a monotherapy has reduced over recent years, MF is still an important alternative in VL chemotherapy, especially when used in combination with other drugs.

ARTICLE HISTORY
Received 30 July 2019
Accepted 13 March 2020

KEYWORDS
Alkyl-phosphocholine; clinical pharmacology; *Leishmania donovani*; *Leishmania infantum*; mechanism of action; miltefosine; pharmacodynamics; pharmacokinetics; preclinical pharmacology; visceral leishmaniasis

1. Introduction: visceral leishmaniasis (VL) and its treatment

The leishmaniases are a group of vector-borne diseases with a wide clinical spectrum caused by approximately 20 different species of an obligate intracellular protozoan parasite [1]. In mammalian hosts, the promastigote form is taken up by resident tissue macrophages and replicates as intracellular amastigotes, which will trigger a persistent immunoinflammatory response [2]. Parasites of genus *Leishmania* may cause two main clinical forms of the disease: VL and cutaneous leishmaniasis (CL) that depend upon the immune status of the patient and the species of the parasite. CL, also known as tegumentary leishmaniasis, has clinical variations that are classified as localized, disseminated, diffuse and mucocutaneous leishmaniasis [3].

Post-kala-azar dermal leishmaniasis (PKDL, caused by *L. donovani*) is a dermal complication characterized by skin lesions, especially in areas exposed to sunlight, that appears weeks or even years after VL has been cured. Treatment of these patients is also required, since PKDL cases are a reservoir for VL and may potentially infect new patients through the insect vector [4]. Most of the cases of PKDL occur in East Africa and Southeast Asia [3].

Closely associated with poverty, the leishmaniases are considered by the World Health Organization (WHO) to be a NTD, endemic in 98 countries and three territories [1], representing one of the largest disease burdens with over 2.4 million disability-adjusted life years (DALYs) and a huge impact on public healthcare [2]. To this date, the leishmaniases remain underreported, lacking simple and effective tools for case management [5]. Potentially fatal and endemic in 75 countries, VL or kala-azar is caused by *Leishmania donovani* (in the Indian subcontinent and Africa) and *Leishmania infantum* (syn. *Leishmania chagasi*) (in the Mediterranean basin, and Central and South America) [6]. In the last five years of data, 168,906 autochthonous human cases have been reported globally [7].

Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan are responsible for almost 90% of all VL cases [7]. In the Americas, 59,769 new cases were reported between 2001 and 2017, 96% of which were concentrated in Brazil. There was a decrease in the number of cases in Paraguay and Colombia, but an increase in Central America, and an overall geographic expansion of the disease globally. In addition, a reduction in the proportion of co-infected HIV-VL cases was observed in 2017 in comparison to the previous year [8]. Despite the known limitations and change in risk patterns (urbanization and domestic transmission) [2], an approximately 50% decrease in the total number of global VL cases has been observed since 2009 [7].

Treatment for VL relies on chemotherapy, this being the most effective but still limited approach. There are very few drugs available, all of which have serious limitations, such as high cost, potential toxicity, long and inconvenient treatment regimens,

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Article Highlights

- VL is a Neglected Tropical Disease (NTD) that is highly associated with poverty, is fatal, and still represents a huge burden to public healthcare, especially in developing countries. Before the introduction of MF, treatment options were all parenterally administered as part of long and expensive schemes with significant side effects;
- MF was initially considered as a potential drug in the fight against cancer, but clinical trials revealed low efficacy and tolerability. Since 1982, several studies have demonstrated MF antileishmanial activity and in 2002 it was registered in India as the first oral antileishmanial drug for VL;
- MF, an alkyl-phosphocholine, is a drug that interferes in phosphatidylcholine and phosphatidylethanolamine synthesis due to a reduction in the intracellular choline, thus affecting the composition of the parasite's plasma membrane;
- Several clinical trials demonstrated MF safety and efficacy against VL and tolerability was high among HIV-coinfected patients. Different levels of response have been reported with an increased risk of relapse in children and males after treatment with MF;
- The decreasing efficacy of MF and the possibility of development of resistance to the drug is an increasing concern. Therefore, combination therapy is clearly needed and clinical trials are required to address this issue;
- MF is an important advance in VL treatment and is an example of successful research and development for this NTD.

notable side effects and drug resistance [9,10]. Pentavalent antimony (Sb^v), amphotericin B (AmB), paromomycin (PM), pentamidine, miltefosine (MF) and some new combinations forms the limited therapeutic arsenal for treatment of VL [11]. One first-line drug for treating VL is Sb^v (except in the Indian sub-continent), presented as sodium stibogluconate (SSG) or meglumine antimoniate (MA). The recommended first- and second-line drugs used in the Indian sub-continent are MF and AmB, respectively, while SSG was completely abandoned in that region in 2005 due to drug resistance [11,12].

MF, the focus of this study, was the first oral antileishmanial agent registered for VL [1]. In 2011, it was added to the WHO's Model List of Essential Medicines [4] and was considered to be a crucial agent for the success of the elimination agenda, as it allows ambulatory treatment of many patients simultaneously [13]. In 2014, the US FDA approved MF registration for leishmaniasis [14]. Adherence to the treatment must be ensured in order to avoid the selection of resistant lines of the parasite [10]. The Food and Drug Administration (FDA) of the United States recommended dosage of MF is: one 50 mg capsule twice daily for 28 consecutive days (for people weighing 30 to 44 kg) and one 50 mg capsule three times daily for 28 consecutive days (for people weighing 45 kg or greater). The most common side effects of MF include: nausea, vomiting and diarrhea. Other side effects include abdominal pain, decreased appetite, dizziness, headache, sleepiness, skin itching, and abnormalities in liver or kidney tests [6]. Since 2006, MF has also been used in the treatment of PKDL in longer-course regimens, ranging from 6 to 16 weeks, with an estimated cure rate of up to 90% [4].

2. Discovery and preclinical development of MF for VL

Ether lipids, as the alkyl-lysophospholipids (ALPs), have several biological functions as important components of mammalian cells and are structurally related to the 'platelet aggregation factor' (PAF), which has demonstrated itself to be a potent phospholipid inflammatory mediator and has been implicated in a great variety of pathophysiological disorders. Several analogs of PAF with an absence of platelet aggregating activity have been synthesized, such as the alkyl analogs of lysophosphatidylcholine (LPC) (Figure 1(a)), which has immunomodulatory properties [15].

In the 1960s, the glycerol-linked ester bond at position C1 of LPC was replaced by an ether linkage and another ether-linked methyl group was added at the C2 position. The resulting ALP, edelfosine (Figure 1(b)), showed a potent immune modulator effect and potent antitumor activity (reviewed by [16]). However, its clinical application has been limited due mainly to its metabolic instability and low selectivity for tumor cells, high hemolytic potential, and gastrointestinal toxicity (reviewed by [17]). Although the clinical use of edelfosine has been limited, these findings prompted a series of chemical and biological experiments aiming to improve the next generation compounds. New analogs were developed, such as ilmofosine (Figure 1(c)); however, this compound did not improve the metabolic stability or reduce the toxicity to the gastrointestinal tract, with no clinical activity in patients [17].

In the mid-1980s, MF (Figure 1(d)) was synthesized as a new type of anticancer agent. The compound, also named hexadecylphosphocholine, was identified as the minimal structural requirement for the antitumor activity of alkyl-phosphocholines (APCs), which differ from alkyl-lysophosphocholines through the lack of a glycerol motif [15,18].

The first studies demonstrated the outstanding antitumor activity of MF in induced rat mammary tumors and a complete suppression of tumor growth after daily oral treatment at 46.4 mg/kg/day. Interestingly, MF showed absence of effectiveness against induced-sarcoma in rats, indicating the drug's tendency to act selectively [15]. Besides this, chemotherapy with MF at low doses resulted in antitumor efficacy and low toxicity [19]. Some signs of toxicity were enteritis, increased reversible transaminase and splenic hemosiderosis [20]. Further distribution and metabolism studies of radiolabeled MF in mice demonstrated that the drug is well absorbed from the intestinal tract [21] and is a substrate for a phospholipase C or related enzyme [22]. MF was then selected for oral treatment of solid tumors and for the topical treatment of cutaneous metastases in patients with breast cancer [23,24]. After successful clinical development, the topical treatment was approved in several countries in Europe as Miltex®, being the first anticancer agent which was specifically formulated for topical use against cancer [25,26]. However, its development as an oral drug was hampered by the gastrointestinal toxicity. MF derivatives were sought with a better therapeutic index and perifosine was identified as a suitable candidate with an enhanced gastrointestinal tolerability and increased anticancer activity [27].

The first report of ether lipid derivatives in *L. donovani* promastigotes was in 1982, when the authors described ether lipid biosynthesis in the parasite. *Leishmania* promastigotes contains significant amounts of ether lipids, especially ethanolamine plasmalogen [28]. The biosynthetic pathway of ether lipid formation in *Leishmania* parasites was found to be similar to that in mammalian cells, whilst long-chain alcohols,

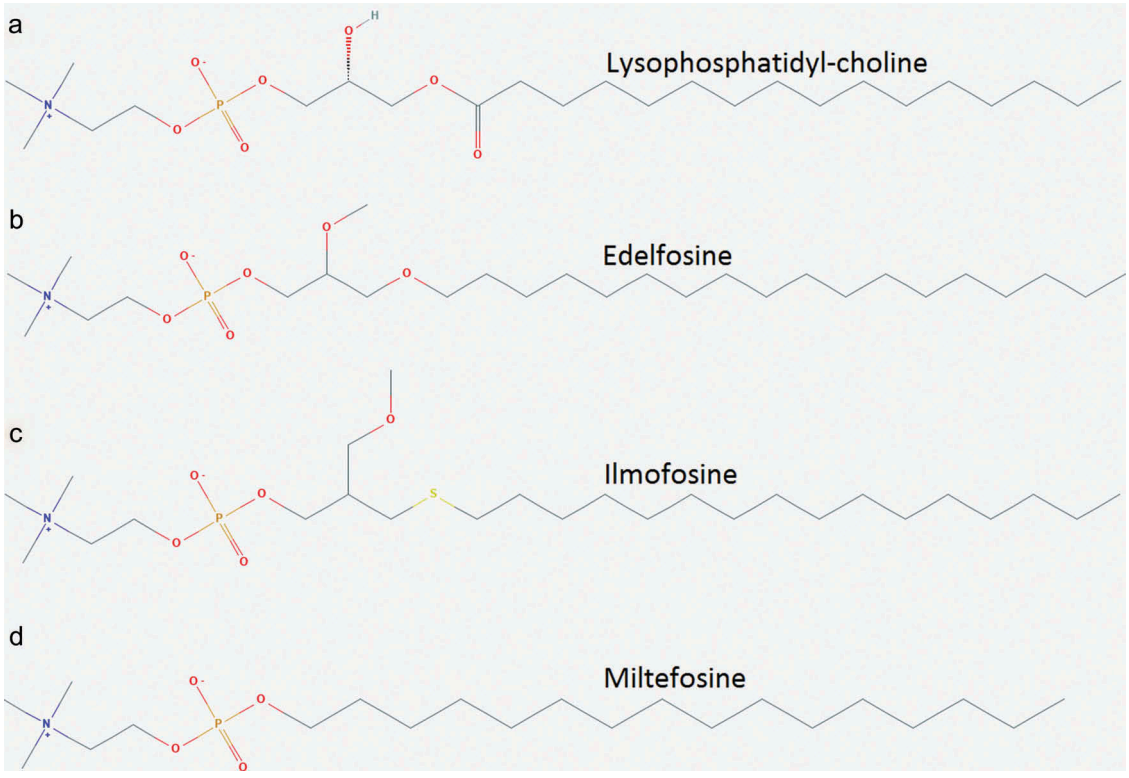

Figure 1. Chemical structure of MF and other alkyl-lysophospholipids.

as well as 1-O-alkyl-glycerols, were shown to be direct precursors [29]. It was demonstrated that 1-O-alkyl-glycerols were toxic to *L. donovani* promastigotes. These compounds caused the rounding of the cells and accumulation of cellular debris, possibly due to the disorganization of cytoskeleton as well as inhibition of cellular processes such as lipid metabolism [29]. Therefore, it was concluded that 1-O-alkyl-glycerols and other lipid analogs could be tested as therapeutic anti-*Leishmania* agents, designed to inhibit the lipid metabolism of the intracellular parasite [29]. Four ester lysophospholipids, three ether lysophospholipids, and two radylglycerols were evaluated against *L. donovani* promastigotes and it was demonstrated that the sensitivity of the parasite toward these ether lysophospholipids was in the micromolar range and was similar to that reported against tumor cells [30]. The highest activity found was the ALP analog edelfosine, which was 10-fold more toxic than the others. However, the authors highlighted that the effect of ether lysophospholipids on the amastigote form should be investigated to determine whether ether lysophospholipids might be useful for the chemotherapy of VL [30].

The *in vitro* antileishmanial activity of another APC derivative, perifosine, against *L. donovani* promastigotes [30] and amastigotes [31] was subsequently reported in 1987. At this time, seven APC including MF and one alkyl-phosphoethanolamine were tested against *L. donovani* intracellular amastigotes, resulting in Half Inhibitory Concentration (IC_{50}) values in the range of 1–12 μM. MF showed an IC_{50} value at the micromolar range against intracellular amastigotes, which was not the lower IC_{50} against the parasite, but the less toxic toward mammalian cells. Besides this, three selected APCs among four were effective against *L. donovani* infected BALB/c mice, and MF showed an ED_{50} of 12.8 mg/kg body weight after 5 administrations by a subcutaneous route [31]. After few years, it was demonstrated the efficacy of MF against an established *Leishmania* infection through 5 days of oral therapy (20 mg/kg of body weight/day) in mice infected 6 weeks before treatment and examined 3 days after the end of treatment. MF led to parasite burden suppression in the liver and the spleen of 94% and 78%, respectively (versus 85% and 55% suppression in the liver and spleen, respectively, by Sb^{V}) [32]. Next, the efficacy of MF was evaluated against an established infection in *L. infantum*-infected BALB/c mice after 10, 31 and 52 days of the treatment, resulting in a reduction of at least 89% of the parasite burden in the liver and spleen. Therefore, MF was shown to be highly effective against *Leishmania* for at least 7 to 8 weeks of following up [33].

Although MF was not the most active compound of its class against *Leishmania in vitro*, its excellent bioavailability and the early demonstration of its activity after oral administration in VL-infected animals boosted its development through a collaboration between the pharmaceutical industry ASTA Medica (later Zentaris) and the WHO/Special Programme for Research & Training in Tropical Diseases (TDR) [26].

The subsequent Phase I/II dose trial to treat VL in Indian men was initiated in 1997. The oral treatment with MF at 100–150 mg/day for 4 weeks proved to be an effective oral treatment [34]. These promising results warranted further testing of MF to determine its potential as an orally administered treatment in VL and opened the way for the further clinical development of MF in an unique collaboration between the pharmaceutical industry, WHO/TDR and the Indian

Government, which led to the approval of MF as the first oral treatment for VL under the trade name of IMPAVIDO™ [26].

Further experimental studies demonstrated that MF is also effective in a Severe Combined Immunodeficient (SCID) mouse model infected with *L. donovani*, in contrast with the lack of activity of Sb^v, suggesting that MF could be effective in HIV co-infected patients [35]. The preclinical antileishmanial activity of MF against several *Leishmania* species was verified, both *in vitro* and *in vivo* [36–41]. In general, these studies demonstrated that the *in vitro* activity of MF differs depending on the *Leishmania* species. *Leishmania braziliensis*, for example, has the lowest MF susceptibility when compared with other *Leishmania* species [42], although a huge variation has been found among Brazilian clinical isolates [43]. This species has IC_{50} values three to 20-fold higher than *L. donovani* amastigotes *in vitro* [44].MF has also proven to be effective against Brazilian canine VL [45], however, further controlled clinical trials are needed in such endemic areas [46].

3. Clinical development of MF for VL

The first results of a pilot Phase II clinical trial of oral MF for VL were published in 1998 [34]. The study reported that 29 of 30 Indian patients who received 50 to 250 mg/day of MF were apparently cured by the end of the treatment. The study highlighted that MF treatment at 100–150 mg/day for 4 weeks was an effective oral treatment for VL, including cases of patients' unresponsiveness to Sb^v treatment.

A Phase II clinical trial was conducted with 120 patients receiving 50 to 150 mg/day MF for 4 to 6 weeks [47], resulting in a cure rate of about 95%. According to this study, oral administration was well tolerated and effective against VL in Indian patients. Another Phase II clinical trial for VL was performed with 45 patients, with Sb^v treatment having previously been unsuccessful with 17 of them. The results indicate that 100 mg/day of MF for 28 days was a promising oral-treatment regimen for VL cases, including those with Sb^v-unresponsive infections, reaching effectiveness of about 95% [48].

A Phase III clinical trial was performed with 299 Indian patients, aged 12 or older, who received 50 or 100 mg/day of oral MF for 28 days [49]. Six months after the completion of treatment, 94% of the treated patients had not relapsed and were classified as cured. Following this Phase III trial, MF was registered in India in 2002 as the first oral antileishmanial drug for VL. The efficacy and tolerability of MF for childhood VL in India were also evaluated in 2004 [50]. The authors evaluated the use of 2.5 mg/kg/day MF for 28 days in 80 Indian children (aged 2–11 years) with a final cure rate of 95%. This trial indicated that MF was as effective and well-tolerated in Indian children with VL as in adults, suggesting that it could be used as the first-line drug for treatment of children with VL in India. Ten years later, a follow up of 1,016 patients showed a high relapse rate (12.8% of per protocol treatment) [51]. Cases of relapse were twice as common among male patients and two to three times more frequent in children compared to people over 25 years of age. These findings demonstrated a direct correlation between host-related factors (particularly age and gender) and an increased risk of VL relapse after MF treatment [51].

A Phase IV trial of MF for the treatment of VL was an open-label, single-arm study under field conditions in 13 clinics in Bihar, India [13]. This study was conducted among 1,132 adult and pediatric VL patients. The results were promising and supported the use of MF in an outpatient setting in an area where VL is endemic. Compliance was good, with 95.5% of patients completing the full 28-day course of treatment. The final cure rate was 82% by 'intention to treat' analysis and 95% by 'per protocol' analysis in a 6 months follow up [13]. Adverse events to the treatment were related to gastrointestinal toxicity and occurred in about 3% of patients. Additionally, another multicentric study reported that when 64 children, including 38 males, were treated with MF, the failure rate among those monitored for 6 months was only 3% [52,53].

A Phase IV trial of MF in 977 adults and children for treatment of VL was performed in Bangladesh, achieving the final per protocol cure rate of 85% [54]. In this trial, treated patients presented 13 severe adverse events and nearly all non-serious adverse events were gastrointestinal. Therefore, oral MF was considered an attractive alternative to intramuscular Sb^v and intravenous AmB for the treatment of VL in Bangladesh.

The efficacy of MF for treatment of VL in an Ethiopian population with a high prevalence of HIV infection was evaluated and compared with Sb^v [55]. A total of 580 patients with VL received oral MF (100 mg per day for 28 days), resulting in an initial cure rate of 88%, although the mortality during treatment was 2% in the MF group. The authors concluded that MF treatment was as effective as Sb^v in non-HIV-infected VL patients, while among HIV-coinfected patients, the drug was safer but less effective than Sb^v [55]. Most recently, a Phase II trial in 30 children with VL, aged 4–12 years, was conducted in Eastern African to test whether 28 days of allometric MF dosing safely achieves a higher systemic exposure than linear dosing. This study indicated that median areas under the concentration-time curve from days 0–210 and plasma maximum concentration values were slightly higher than those reported previously for children on linear dosing, but not dose-proportionally. Drug exposure at the start of treatment was increased, with higher median plasma concentrations on day 7, while concentration-time curves were less variable, avoiding the low levels of exposure observed with linear dosing. The achieved cure rate was 90%, similar to that previously described in adults [56].

A Phase II dose-ranging trial to assess the efficacy and safety of orally administered MF in patients with VL in Brazil monitored 42 patients. The patients were treated with 2.5 mg/kg/day of MF for 28 days (14 patients) or 42 days (28 patients) and they were monitored for at least 6 months, but at most 1 year after treatment. This trial revealed a cure rate of approximately 60%, which is lower than that found for Indian VL when MF was first used in India (>90%). The cure rate between the pediatric and adult patients did not exhibit a significant difference (treatment failure rate of 52.2% and 26.3% in pediatric and adult patients, respectively) [57].

MF was initially developed by Asta Medica (later Zentaris), in close cooperation with WHO/TDR. Currently, it is manufactured by Paladin (Quebec, Canada), after the rights to production and marketing were obtained from Zentaris [58]. However, the future of MF is uncertain, considering the access

problem: the current price of MF is relatively high, its demand is low, and its production is irregular, among other issues that need attention, such as lead times for orders and possible low stock from the holder [58].

4. Post-launch data and additional studies

4.1. Pharmacokinetics and pharmacodynamics

Investigations into the clinical pharmacokinetics of MF were limited before the drug was approved for the treatment of VL [59]. After clinical development, some studies described the plasma concentrations of the drug in VL [60,61] and CL patients [62,63], providing some information about their clinical pharmacokinetics. After oral administration, MF achieves maximum concentration between 4 and 48 h, in a slow but complete absorption [64] in animals (rats and dogs), with an absolute bioavailability (which has never been assessed in humans) of 82%-94%. The estimated gastrointestinal absorption rate was 1.67 h in a two-compartment pharmacokinetic model [59], taking at least 2 weeks to reach therapeutic blood levels [4,59]. MF preferentially binds to serum albumin and to a lesser extent to low-density lipoprotein. Human plasma protein binding of MF, evaluated by an ultracentrifugation method, was 98% over the drug concentration range from 0.1 to 10 µg/mL [65]. MF presents wide distribution and high accumulation during treatment, showing especially high levels of the radio-labeled drug in the kidneys, intestinal mucosa, liver and spleen (in that order) [21,64]. Steady-state concentrations could be achieved in all organs and serum except for the kidneys and brain. The extent to which MF penetrates the brain remains unknown, since it was only demonstrated in rats, but has never been evaluated in humans [66]. The highest drug concentrations were observed in the adrenal glands, kidneys, spleen and liver when a steady-state oral unlabeled MF dose was administered in a subsequent study in rats [59]. Transfers via the placenta or the umbilical cord have not been studied yet, but findings indicate that this occurrence is possible [67].

The pharmacokinetic parameters for MF following oral capsule administration to adolescents (≥12 years) and adult patients with VL differ according to the regimen adopted. In a 50 mg dosage of MF taken two times a day for 4 weeks (mean dose per kg of 3.1 mg/kg/day), the maximum plasma concentration (C_{max}) achieved was 66.2 µg/mL and the distribution phase half-life ($t_{1/2,\alpha}$) was 6.4 days on day 23. Meantime, in a 50 mg dosage of MF taken two times a day for 1 week or in a dosage of 50 mg of the drug taken three times a day for 3 weeks (mean dose per kg of 3.6 mg/kg/day), the achieved C_{max} was 75.9 µg/mL and the $t_{1/2,\alpha}$ was 8.5 days. Besides, due to the long half-life of MF (>6 days), trough plasma concentrations did not appear to reach a steady state at the end of treatment (i.e. Day 28) [65].

The degradation of MF is mediated by phospholipases C or D, releasing hexadecanol and choline, respectively [21,59]. Very little intact MF is excreted; rather, it is extensively metabolized. Hexadecanol can be oxidized to palmitic acid and enter lipid biosynthesis or beta-oxidation, whilst choline is later used for the synthesis of acetylcholine or lecithin [30,64]. Since it does not show interaction with cytochrome P450 (CYP) isoenzymes, inhibition or induction of other drugs by these systems is not expected [67]. Almost completely eliminated by its metabolic mechanisms, MF has an extremely slow plasma clearance, a primary elimination half-life of approximately 7 days and a terminal half-life of 31 days, estimated from a two-compartment pharmacokinetic model [62]. Fecal excretion is not expected due to its long elimination half-life. The excretion into breast milk was not investigated in humans, but maybe expected to occur [59,67].

The gastrointestinal system is the primary site for (dose-limiting) side effects [67] such as loss of appetite, mild nausea, vomiting and diarrhea, which can lead to a fluid imbalance and rehydration may be necessary in severe cases [59]. This imbalance, in addition to the direct impact of MF on the kidneys, may contribute to an increase in levels of creatinine [64]. The severity of these side effects decreases with food intake and during treatment [59]. Gastrointestinal manifestations, hepatotoxicity and nephrotoxicity are the main side effects, which may increase therapy costs, since it requires monitoring [68]. A mild increase of serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) may be observed in the first week of treatment, normalizing in the subsequent weeks [47,59,69]. Since there is a risk of embryotoxicity, fetotoxicity and teratogenicity, the use of effective contraception for the duration of treatment and for another 2–4 months after the last dose is recommended [59,64,68]. The FDA informs that the drug may cause fetal harm and warns that fetal death and teratogenicity occurred in animals treated with MF at doses lower than the recommended human dose. The prescribing information of MF also highlight to not administer the drug to pregnant women and to obtain a serum or urine pregnancy test in females of reproductive potential prior to prescribing the drug and advise females of reproductive potential to use effective contraception during therapy and for 5 months after therapy [65].

MF promotes a rapid increase in leukocytes and platelets at the beginning of the treatment, which can be beneficial for patients with VL who usually have pancytopenia [64,70]. No side effects regarding nervous, respiratory or hematological systems were reported. At the recommended dosage, there are no ophthalmologic repercussions, the risk of nephrotoxicity is limited, and any negative impact on male fertility is not expected [64], but this is currently under assessment by the FDA [4]. Usually, adverse effects are more pronounced in the first week of treatment, decreasing with time (even though drug levels increase), probably due to the diminished parasite burden [13,71].

4.2. Mechanism of action and resistance

The mechanism of action of MF against the parasite is not completely understood. However, over the past two decades, some studies have shown that the drug affects the parasite membrane composition by inhibition of phospholipid metabolism. In *L. donovani*, a concentration of 10 µM MF interferes mainly in phosphatidylcholine and phosphatidylethanolamine synthesis, affecting the content of these two phospholipids in the plasma membrane of the parasite [72]. For the synthesis of these phospholipids, it is required the transport of choline precursor from the host that is inhibited by MF, affecting, therefore, the intracellular accumulation of choline and

consequently the synthesis of these phospholipids. [59,73] The content of sterol is also affected by a drastic reduction of the C_{24} alkylated sterol content and an increase of the cholesterol content due to a condensation effect of the drug with sterols [72]. In addition to these changes, an increase in the production of reactive oxygen species (ROS) has been reported in wild-type promastigotes treated with the drug [74,75] that can be explained by a decrease in metabolites as arginine, ornithine and citrulline involved in the biosynthesis of arginine-polyamines pathway that produces spermidine for biosynthesis of trypanothione [76,77], the main anti-oxidant of trypanosomatids against oxidative stress [78]. MF also inhibits enzymes as cytochrome-c oxidase and superoxide dismutase, affecting the electrons transport chain and ROS detoxification in the mitochondria, respectively [74,79]. These findings corroborate the significant reduction in mitochondrial membrane potential in promastigotes of *L. amazonensis* exposed to 10 μM of MF [80]. More recently, in one study on untargeted metabolomics have confirmed remarkable alteration in the metabolism of lipids in *L. donovani* axenic amastigotes that is characterized by a significant reduction in membrane phospholipids and an increase in sterols and sphingolipids levels in treated parasites [81].

Studies using promastigotes resistant to MF (maintained by continuous selection of 40 μM MF) have shown that the drug leads to a reduction in ergosterols levels and changes in the length and levels of unsaturated fatty acids [82,83]. Unlike mammalian cells, *Leishmania* spp. synthesizes ergosterol as an essential component of plasma membrane [84]. The depletion of sterols decreases MF susceptibility in wild-type and resistant lines of *L. donovani* [83], indicating that the plasma membrane is an important target of the drug. Similarly, in other cell lines MF also affects lipid metabolism [85,86].

There are two main routes for the drug to be internalized by the parasite, by binding to the outer leaf of the plasma membrane and then being endocytosed, or by a flippase activity mediated by a transporter of phospholipids, known as the MF transporter (MT) [87]. The MT forms a complex with a non-catalytic subunit, Ros3 that is mainly responsible for phosphatidylcholine accumulation by a process dependent on ATP mediated by the MT-Ros3 complex [88,89]. This machinery is essential for drug internalization and consequently drug activity against the parasite. Recent studies have demonstrated a correlation between MF accumulation and susceptibility to the drug [38,42,87,90,91]. Moreover, this complex is also involved in maintaining the phospholipid asymmetry of the parasite membrane [92]. Once internalized, the drug may be eliminated by exocytosis or by floppase activity that is mediated by ABC transporters. Two members of the ABCG subfamily and one member of the ABCB subfamily were already involved in the transportation of MF from the cytosolic to the exoplasmic face [93–95].

Although reports of MF resistance in the field are restricted, promastigotes of *Leishmania* may be easily selected *in vitro* by drug selection (stepwise manner) [38,87,96] or by chemical mutagenesis followed by drug selection [87,97,98]. The main mechanism involved in MF resistance is related to a defect in drug internalization mediated by the MT-Ros3 complex due to mutations in the coding sequences of the *MT* gene [38,87,96,99,100], and *Ros3* gene [88]. These findings indicate that both proteins are selected during drug pressure, although mutations in the *MT* gene are more recurrent than in the *Ros3* gene in resistant parasites [42,87,96,100,101]. Interestingly, inactivation of the *MT* gene leads to a MF resistance phenotype that persists in amastigotes *in vitro* and in infected animals with species responsible for both forms of the disease (VL and CL) [38,102]. These findings demonstrate that the MT-Ros3 complex is functional throughout the life cycle of the parasite and is essential for MF effectiveness against the parasite. In the field, cases of relapses after successful MF treatment have been reported for CL, diffuse CL and VL [103–109]. These reports may be associated with a decrease in the susceptibility of parasites to MF. A restricted number of studies have shown the selection of resistant lines after treatment with MF [101,110]. In one of these studies, a description was provided of the selection of a *L. infantum* resistant line after treatment with MF in a HIV-coinfected patient and the mechanism involved was associated with the occurrence of a mutation in the *MT* gene [110]. In another study, mutations in the *MT* and *Ros3* genes were associated with MF resistance in a clinical isolate of *L. infantum* from a patient who had received successive treatments with AmB and MF [101]. A decrease in MF susceptibility was also found in clinical isolates of *L. donovani* after MF treatment of patients with VL and PKDL [111,112], although mutations were not found in the genes that code for the MT-Ros3 complex [111]. In contrast, in patients infected with *L. donovani*, VL relapse cases were found in up to 20% after 6–12 months, but none of the isolates from these patients were resistant to MF *in vitro* [106]. Similar findings were found from isolates of *L. donovani* from cured and relapsed patients [113]. More recently, a significant association between the presence of one *locus* in chromosome 31, named MF Sensitivity *Locus* (MSL), was demonstrated, with the effectiveness of the treatment of VL due to *L. infantum*. A ten-fold risk of treatment failure was found when the clinical isolate did not have this *locus* [57]. This *locus* has four genes in chromosome 31 but these genes are not correlated with previous drug resistance genes described in MF resistant lines selected *in vitro*. Later, it was demonstrated that MF susceptibility *in vitro* of the clinical isolates from cured and relapsed patients had a positive correlation with the clinical response [114]. In addition, a positive correlation was also found between the presence of the MSL *locus* and the susceptibility to MF (mean IC_{50} = 5.9 μM; SEM = 1.0 μM), while MSL negative isolates were less susceptible to the drug (mean IC_{50} = 10.9 μM; SEM = 1.8 μM), corroborating the previous association between MSL *locus* and the clinical response [114]. Further studies are necessary to investigate the frequency and distribution of MSL *locus* in *L. infantum* populations in Brazil, the absence of which is directly correlated to the natural resistance of this species.

The activity of the drug against the parasite depends on pharmacokinetics and pharmacodynamics in patients with VL, but also on MF susceptibility of these parasites. The lower exposure of drug in children compared to adults may be responsible for the low efficacy of the drug in these patients in Southeast Asia [51,115]. In this endemic region, the initial cure rate of MF in VL due to *L. donovani* was higher than 90%, but in recent years an increase in the number of

relapses after the end of treatment has been reported (approximately 10% and 20% have been reported in India and Nepal, respectively, after more than 10 years of use) [106,116]. These relapses were not due to intrinsic or acquired resistance of the parasites after MF treatment [113] and others factors unrelated to drug resistance may be responsible for the failure of the treatment, these including host-related factors (i.e. immunological factors), infectivity of the clinical isolates, and/or a low exposure of the parasites to the drug as mentioned before [51,115,117–119]. Rather, some studies have also observed an increase in MF resistance *in vitro* after treatment of the patients with the drug [111,112]. An increase in MF tolerance may be due to MF underexposure of the recommended dose, as observed in children in Southeast Asia [51]. Although this restricted number of drug-resistant isolates has been reported, the decreased efficacy of MF in the last years may increase the possibility of selection of drug-resistance parasites. In addition, the long half-life of MF (around 120 h) may also favor the selection of resistant lines in endemic regions where MF is used [120]. The parasitic factors involved in MF unresponsiveness in clinical isolates of *L. donovani* are apparently similar in laboratory adapted parasites: the parasites isolated from the cases that relapsed exhibited high infectivity, increased metacyclogenesis, reduced drug accumulation and reconfigured metabolism to overcome the oxidative stress induced during drug exposure [121].

4.3. Multidrug therapy

It has been questioned whether the standard monotherapy regimen should be replaced by a combination of different drugs, which could impact the outcome of treatment and avoid the selection of drug-resistant lines of the parasite [122]. Multidrug therapy may prevent *Leishmania* from acquiring resistance to the drugs [122] and could be a more effective strategy, with lower dosages and fewer side effects in a shorter-course treatment [1].

The rationale for drug combination therapy includes reduction of the course of treatment, reduction of toxicity, and increasing compliance and adherence of the patients, hence prolonging the drugs' therapeutic lifespans [123,124]. In addition, drug combination may also prevent the selection of drug-resistant parasites [123,125]. Therefore, clinical trials should be conducted in order to discover which combinations will present synergistic or additive effects on different targets [6], thus increasing antileishmanial activity. Although highly effective, MF used as a monotherapy must not be encouraged in large scale programs. However, MF should be considered as a potential candidate for a multidrug combination regimen [10].

Short course combination regimens with AmB, MF and PM for the treatment of VL were evaluated in a Phase III clinical trial in Bangladesh [126]. The study aimed to compare the safety and efficacy of the follow regimens: a 5 mg/kg single dose of AmB plus 7 subsequent days of MF (2.5 mg/kg/day), a 5 mg/kg single dose of AmB plus 10 subsequent days of PM (15 mg/kg/day), and 10 days of PM (15 mg/kg/day) plus MF (2.5 mg/kg/day), and a standard regimen of AmB 15 mg/kg given in 5 mg/kg doses on days 1, 3 and 5. Results indicated that none of the combinations were inferior to AmB alone and all the combinations demonstrated excellent overall efficacy, and were well tolerated and safe [126].

MF (9, 3 and 1 mg/kg) *in vivo* activity was enhanced when the drug was co-administered, in a preclinical study, with Liposomal AmB (L-AmB) (5 mg/kg) or PM (63 mg/kg), with no signs of toxicity being recorded. On the other hand, the combination of MF and Sb^v showed no increase in effectiveness in *L. donovani*-infected mice [59,127]. In a Phase III clinical trial, a single injection of L-AmB (5 mg/kg) followed by 50 mg of MF for 7–10 days, once or twice a day, according to body weight, reduced treatment duration without decreasing drug efficacy (definitive cure rate) [124]. Other regimens have been tested, all of them effective and less toxic (especially for the kidneys) and final cure rates were high (>95%) and similar in all the groups [6,124]. A single infusion of L-AmB followed by a brief self-administered course of MF could be an excellent option against Indian kala-azar [128], but when considering the toxicity profile and treatment cost, PM appears to be a better partner drug to MF [127]. However, using two self-administered oral drugs would be the ideal scenario [128]. The estimated cost of standard treatment with L-AmB is $436 (considering the drugs and hospitalization), while multidrug therapy costs range from $71 to $126, depending on the combination (WHO-negotiated prices) [124,129]. Given the fact that MF may be administered in-home, while patients must travel to clinics to receive the first-line parenteral drugs (as Sb^v), a cost-effectiveness analysis comparing both treatments indicates that MF treatment is cost saving from both patient and societal perspectives [130].

These days, a single dose of L-AmB and multidrug therapy (L-AmB + MF, L-AmB + PM or MF+ PM) is the recommended regimen for treatment of VL in the Indian sub-continent [131,132]. The combination of therapies using MF are well tolerated and effective options and are associated with shorter duration of hospitalization and are an excellent option for treating VL [68].

5. Conclusion

MF is an anti-leishmanial drug that was originally developed in the 1980s as an anti-cancer agent. It is currently the only recognized oral agent used to treat VL and CL. Several potential MF antileishmanial mechanisms of action have been proposed, although the mechanism of action was not completely elucidated. Little is known about the clinical pharmacodynamics of MF. Its use during pregnancy is strictly contraindicated, and contraceptive use is mandatory for females of child-bearing age during therapy and the following months. An increase of unresponsive and relapse cases of VL patients treated with MF has been reported, indicating an urgent need for alternative drugs and/or a multidrug combination using MF as a potential partner. A summarizing timeline demonstrating the main events in the course of MF discovery and development for VL is shown in Figure 2.

6. Expert opinion

MF, the only oral drug approved for the treatment of VL, is an example of successful research and development for a NTD, as its oral administration has enabled more patients to be treated

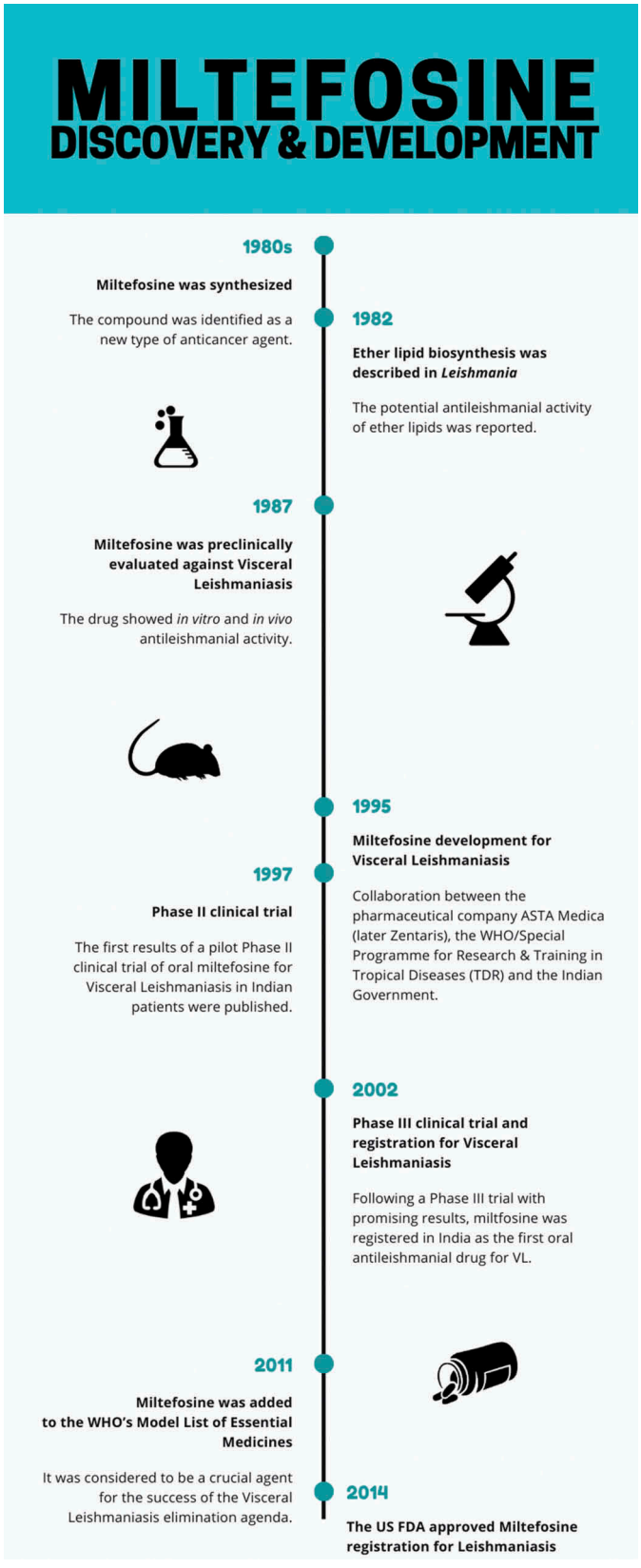

Figure 2. Summarizing timeline demonstrating the main events in the course of MF discovery and development for VL.

in primary care settings. However, although some papers describe oral MF as 'well-tolerated,' surely the high incidence and severity of vomiting and diarrhea suggests otherwise and could contribute to a poor patient compliance. The embryo-fetal toxicity is also an important issue, due to the requirement for a pregnancy test before treatment and the need for adequate contraceptive coverage (preferably non-oral contraception due to vomiting) for 5 months after therapy. Besides, cultural and religious beliefs are obstacles to contraception, not to mention the access to and cost of pregnancy testing and adequate contraceptive cover in regions of poverty. These issues effectively exclude treatment with MF for women of childbearing age in some endemic regions. Also, the decline in MF efficacy and a high rate of relapse in VL as well as PKDL, is of great concern. Because of this, MF is no longer widely used in some endemic regions and the preferred first-line treatment has become single-dose L-AmB, which overcomes the main drawbacks of MF as its teratogenic potential and the month-long therapy. However, the treatment with L-AmB also has limitations, such as the need for a cold-chain. Therefore, current limitations of VL treatment have provided the rationale for essential research on combination therapy and the identification of novel classes of chemical entities. The discovery of more effective drugs for VL should be a priority and one that is essential if it is to be successfully eliminated.

It should also be mentioned that almost all clinical trials of MF for VL have been performed in the Indian subcontinent, mainly in the state of Bihar. Other important countries of VL endemicity have not been completely evaluated for MF clinical efficacy and the drug is still not available for human treatment. For the implementation of MF, clinical trials in these areas must be performed considering the species-related variation in the therapeutic response of VL and the geographical variation in such areas. Besides this, some particular concerns must be considered. For example, in Brazil, MF has been recently approved for the treatment of canine VL, although the drug is not yet available for VL human treatment. Although MF has proven to be effective against Brazilian canine VL, there is a potential risk of selection of resistant parasites, thus compromising the possible use of this drug in the treatment of human VL in Brazil.

Faced with the restricted VL therapeutic arsenal, the discovery of MF was an enormous advance, since it was the first oral drug for this neglected disease. Its facilitated administration allows the treatment to be performed without the need to hospitalize the patient. However, its implementation has encountered some obstacles, such as, mainly, teratogenicity and gastrointestinal effects, that compromise adherence to the treatment. Longer treatment regimens and long plasma half-lives favor the selection of resistant parasites. Its major limitation, in our opinion, is the possibility of selection of resistant parasites, which has already been proven *in vitro* and has also been observed in the field, and the growing occurrence of relapses after treatment. Its efficacy as a monotherapy is becoming more reduced, but therapeutic failures have been attributed to the low exposure in pediatric patients. Although other treatments have been proposed in order to replace MF, the drug is still an important option in VL therapy, being associated with another drug, or in HIV patients and pediatric patients and in cases of PKDL, being also useful in the treatment of CL and ML.

Another key issue is the limited access to MF, as a consequence of low production and high prices. Therefore, an agreement between WHO and the manufacturer is necessary to maintain MF production for as long as needed. Considering that MF is not the cheapest drug available for treatment of VL, combination with PM, for example, may also offer a more cost-effective alternative for this neglected disease.

Finally, MF is the only oral drug available for the chemotherapy of VL and it is still highly effective against this form of the disease. Even though a restricted number of reports of treatment failure are correlated with drug resistance, investigation of MF resistance in the field is urgently required. Understanding drug resistance is an essential prerequisite for monitoring the effectiveness of MF and preserving its clinical use. Others aspects, such as host-related factors, underexposure of the recommended dose of MF, virulence, and drug exposure of the parasites, have been correlated to the increased number of cases of treatment failure in patients with VL. These factors must be investigated in more detail in order to understand which aspects are involved in each report of treatment failure in the field. Despite these drawbacks, MF has valuable features including its antileishmanial activity and oral administration whilst it represents a good option for VL treatment in several effective regimens and is already considered to be a partner drug in combination schemes with other antileishmanial drugs. Especially for MF, combination therapy is clearly needed and clinical trials should be designed and performed in this way.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP) [grant number 2016/21171-6 and 2018/18954-4]. D Pita Pedro was financed by a scholarship granted by the Institutional Scientific Initiation Scholarship Program from Faculdade de Medicina de Jundiaí (PIBIC/FMJ). AC Coelho has also, in part, received funding from UK Research and Innovation via the Global Challenges Research Fund under grant agreement 'A Global Network for Neglected Tropical Diseases' [grant number MR/P027989/1].

Declaration of Interest

JQ Reimao and DP Pita Pedro are employee and student, respectively, of the *Faculdade de Medicina de Jundiaí* and while AC Coelho is an employee of the *Universidade Estadual de Campinas*. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer Disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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