CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

204684Orig1s000

MICROBIOLOGY / VIROLOGY REVIEW(S)

Division of Anti-Infective Products
Clinical Microbiology Review

NDA: 204684 (SDN-001, -004, -007, -010) Original NDA
Miltefosine capsules for oral administration
Paladin Therapeutics Inc.

Reviewer: Shukal Bala, Ph.D.
Date Submitted: 9/26/2012; 11/12/2012; 4/19/2013; 6/11/2013
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APPLICANT:
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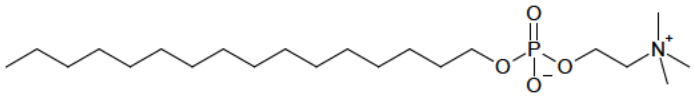
DRUG PRODUCT NAMES:
Proprietary: Impavido®
Nonproprietary: Miltefosine; HePC (hexadecyl phosphocholine)
Chemical Name: 2-[[(hexadecyloxy) hydroxyphosphenyl]oxy]-N,N,N-trimethylethylammonium inner salt

STRUCTURAL FORMULA:



MOLECULAR FORMULA:
$C_{21}H_{46}NO_4P$

MOLECULAR WEIGHT:
407.6

DRUG CATEGORY:
Anti-parasitic/protozoal

PROPOSED INDICATION:
Treatment of adolescents and adults ≥12 years of age weighing ≥30 kg (66 lbs) for treatment of:

- Visceral leishmaniasis due to *Leishmania donovani.*
- Cutaneous leishmaniasis due to members of the *Leishmania (L) viannia (v)* subgenus (*L.v. braziliensis, L.v. guyanenesis, L.v. panamensis*).
- Mucosal leishmaniasis due to *L.v. braziliensis, L.v. guyanenesis, and L.v. panamensis*

PROPOSED DOSAGE FORM AND STRENGTH:
Capsules, each containing 50 mg miltefosine.

ROUTE OF ADMINISTRATION AND DURATION OF TREATMENT:
Impavido is recommended to be taken orally daily for 28 days with food. The number of 50 mg capsules per day will be determined by bodyweight:

- 30–44 kg (66–97 lbs): one 50 mg capsule twice daily with food.
- ≥45 kg (99 lbs): one 50 mg capsule three times daily with food.

DISPENSED:
Rx

RELATED DOCUMENTS:
IND 105,430

REMARKS

The subject of this NDA is miltefosine for the treatment of visceral, mucosal, and cutaneous leishmaniasis. The nonclinical and clinical microbiology studies, submitted by the applicant or obtained by an independent literature search, support the activity of miltefosine against visceral, mucosal, and cutaneous leishmaniasis. A potential for development of resistance to miltefosine exists and may be due increase in drug efflux, mediated by the overexpression of the ABC transporter P-glycoprotein and/or a decrease in drug uptake by the inactivation of the miltefosine transport machinery that consists of the miltefosine transporter and its beta subunit. Mutation in the transporter gene was reported in a relapsed patient in one study. Also, some strains of *L. braziliensis* with intrinsic resistance to miltefosine have been identified. Such information should be included in 'Microbiology' subsection of the labeling.

In clinical studies, the parasitological measurements at the time of screening included direct examination of aspirates/smears; at the end of treatment or follow-up visits parasitological measurements were made if clinically indicated.

The different *Leishmania* species are morphologically indistinguishable. Species may be differentiated by isoenzyme analysis, molecular methods such as polymerase chain reaction, or fluorescent antibody tests using monoclonal antibodies; however, these assays are not FDA cleared tests. In some of the clinical trials for cutaneous leishmaniasis, efforts were made to identify the *Leishmania* species by one of these tests for research purposes. However, the details of the methods and performance characteristics of the assays in the laboratories where testing was performed were not available for an independent review. Therefore, the summary of the clinical trial findings (cure rates) in the labeling should be based on *Leishmania* species known to be prevalent in the endemic areas based on epidemiological findings in different geographic regions and not on *Leishmania* species identified by experimental methods.

The applicant proposes to state the following in the 'Highlight' and 'Indication' sections:

- Visceral leishmaniasis is due to *Leishmania donovani.*
- Cutaneous leishmaniasis is due to *Leishmania (L) viannia (v) subgenus (L.v. braziliensis, L.v. guyanenesis, L. v. panamensis).*
- Mucosal leishmaniasis is due to *L. v. braziliensis, L. v. guyanenesis,* and *L. v. panamensis.*

It is recommended that any reference to *Leishmania* species should be deleted or all the indications should be worded as

- Visceral leishmaniasis in regions where *Leishmania donovani* is known to be prevalent.
- Cutaneous leishmaniasis in regions where *Leishmania braziliensis, Leishmania guyanensis, Leishmania panamensis* are known to be prevalent.
- Mucosal leishmaniasis in regions where *L. braziliensis* is known to be prevalent.

CONCLUSIONS AND RECOMMENDATIONS

From clinical microbiology perspective, this NDA submission is approvable pending an accepted version of the labeling.

The changes proposed in sections 12.1 and 12.4 of the labeling are as follows (additions marked as double-underlined and deletions as striked out):

12.1 Mechanism of action

Miltefosine is an anti-leishmanial agent *[see Clinical Pharmacology, Microbiology (12.4)]*.

12.4 Microbiology

Mechanism of Action

The specific mode of action of miltefosine (b) (4) against *Leishmania* species is unknown. The mechanism of action of miltefosine is likely to involve interaction with lipids (phospholipids and sterols), including membrane lipids, inhibition of cytochrome c oxidase (mitochondrial function), and apoptosis-like cell death.

Activity *In Vitro* and *In Vivo*

Miltefosine has (b) (4) anti-leishmanial activity *in vitro* and in (b) (4) clinical infections [*see Clinical Studies (14)*]. Sensitivity of different *Leishmania* species as well as different strains of a *Leishmania* species to miltefosine may vary in different geographic regions.

Drug Resistance

(b) (4) *In vitro* studies show a potential for development of resistance to miltefosine. Some strains of *L. braziliensis* with intrinsic resistance to miltefosine have been identified. However, the clinical relevance of such an effect is not known.

Drug resistance could be due to a decrease in miltefosine accumulation within *Leishmania* parasites which is thought to be due to either an increase in drug efflux, mediated by the overexpression of the ABC transporter P-glycoprotein and/or a decrease in drug uptake by the inactivation of the miltefosine transport machinery that consists of the miltefosine transporter and its beta subunit. Mutation in the transporter gene was reported in a relapsed patient in one study.

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1. EXECUTIVE SUMMARY

The subject of this NDA is miltefosine for the treatment of visceral, mucosal, and cutaneous leishmaniasis.

Mechanism of action

Miltefosine, an alkylphosphocholine, was shown to interact with lipids (phospholipids and sterols), including membrane lipids, inhibit cytochrome c oxidase (mitochondrial function), and cause apoptosis like cell death. These studies were performed using the promastigote forms of *Leishmania mexicana, Leishmania major, Leishmania donovani, Leishmania panamensis,* and/or *Leishmania infantum.*

Activity in vitro

In vitro activity of miltefosine was evaluated against the promastigote and amastigote forms of a small number of strains of different *Leishmania* species that include *L. donovani, L. infantum, L. mexicana, L. major, L. tropica, L. aethiopica, L. panamensis, L. guyanensis, L. chagasi,* and *L. amazonensis* (Table 1). Based on different studies, the highest 50% inhibitory concentration (IC_{50}) values reported was 10.2 µg/mL against the promastigote forms of *L. donovani* (strain DD8) and 15.2 µg/mL against the amastigote forms of *L. major.* The sensitivity of different *Leishmania* species to miltefosine cannot be compared, based on testing in different laboratories, due to a lack of standardization of the methods. There were inter-laboratory variations in the methods used, source of the strains, and limited number of strains tested. In one study, the activity against the promastigotes and amastigotes of one strain each of 6 different *Leishmania* species were compared for *in vitro* sensitivity to miltefosine; the results suggested *L. donovani* to be the most sensitive species and *L. major* the least sensitive.

Testing of clinical isolates was limited to *L. donovani* species and the IC_{50} values ranged from 0.6 to 7.4 µg/mL against the promastigote forms and 0.01 to 10.9 µg/mL against the amastigote forms. In one study, the 90% inhibitory concentrations (IC_{90}) values ranged from 9.8 – 23.8 µg/mL against the clinical isolates of *L. donovani.* In another study, a trend towards higher IC_{90} values against isolates from high endemic region compared to low endemic area was reported suggesting differences in sensitivity to miltefosine of isolates from different regions.

In vitro sensitivity against the amastigotes is tested using macrophages. However, the role of differences in the source of macrophages in the sensitivity of amastigotes to miltefosine *in vitro* is unclear. In one study, the anti-leishmanial activity of miltefosine against the amastigote forms was similar using peritoneal macrophages derived from either scid or BALB/c mice. However, in another study a trend towards higher IC_{50} values was observed using J774.A.1 cells (mouse cell line) compared to peritoneal macrophages. It is unclear if such differences were due to differences in activation stage of the macrophages or inter-laboratory variability.

A ratio of cytotoxicity to murine macrophages to biological activity against the amastigotes of different *leishmania* species varied from 5 to 420 fold.

Table 1: A summary of *in vitro* activity of miltefosine against *Leishmania* species

Species	Strain	IC_{50} (µg/mL)	
		Promastigotes	Amastigotes
L. donovani	MHOM/SD/00/1S-2D	4.1	-
	MHOM/IN/54/LRC-L.51	2.1 (2.1)*	-
	MHOM/IN/80/DD8	0.9 (10.2)*	-
	MHOM/ET/67/L82	0.15-0.18 (5.5)*	4.7 (1.3 – 1.9)*
	MHOM/ET/67/L82; LV9	-	1.6
	MHOM/IN/82/Nandi II	-	0.08
	Clinical isolates (n=24)	0.6-5.1 (1.1-7.4)*	0.1-10.1 (0.01-2.5)*
	Clinical isolates (n=28)**	-	3.5-10.9
L. braziliensis	WHO-MHOM/BR/75/M2903	-	2.2
L. infantum	MHOM/ES/86/STI-172	2.3	-
L. mexicana	MNYC/BZ/62/M379	1.2-1.3 (5.7)*	-
	MHOWBZ/84/BEL46	5.7	-
	MHOM/BZ/82/BEL21	0.98-5.2	2.8-4.1
L. major	MHOM/SA/85/JISH118	2.0-5.4	12.9-15.2
L. tropica	MHOM/AF/82/KOO1	0.25-0.7	2.4-4.2
L. aethiopica	MHOM/ET/84/KH	0.5-1.1	1.1-2.0
L. panamensis	MHOM/PA/67/BOYNTON	0.5-1.5	4.3
L. guyanensis	MHOM/BR/1997/321-P	-	1.6
L. chagasi	MHOM/BR/70/BH46	-	1.8
L. amazonensis	IFLA/BR/1967/PH-8	-	1.3

* represent results of separate studies
**clinical isolates collected from patients with visceral leishmaniasis or post-kala-azar dermal leishmaniasis in India

Activity in animal models

The studies in murine models acute and chronic visceral leishmaniasis disease show miltefosine to be effective in reducing parasite burden in liver and/or spleen from immunocompetent mice infected with *L. donovani* or *L. infantum.* In one study, the activity of miltefosine was about 3-fold higher against the Indian strain of *L. donovani* compared to the Ethiopian strain. A similar trend in higher activity (lower IC_{50} value) was observed *in vitro.* The activity of miltefosine in immunocompromised mice (scid, T cell-deficient nude mice, IFN-γ gene knockout mice bred on a C57BL/6 background, respiratory burst-deficient $gp91^{phox-1}$ mice that include X-linked chronic granulomatous disease mice and inducible nitric oxide synthase knockout mice lacking macrophage microbicidal mechanisms) was similar to that in normal mice.

Relapse was measured in one study in nude BALB/c mice infected with *L. donovani*; 1 week of miltefosine treatment was effective in suppressing parasites but did not prevent recurrence. However, long term treatment (once or two times a week for 9 weeks) was effective in preventing relapse/recurrence.

In cutaneous disease murine model, topical treatment with miltefosine was effective in reducing parasite burden and lesions in 3 strains of mice infected with *L. mexicana* or *L. major*. Although miltefosine was effective in all the 3 strains of mice infected with *L. mexicana*, parasites were still detectable in the draining lymph nodes from CBA/J mice but not in BALB/c and C57BL/6 mice suggesting variability in treatment effect. This may be associated with host immune response as antigen-specific lympho-proliferation was not observed in CBA/J mice but was observed in BALB/c and C57BL/6 mice. The activity of oral treatment in the cutaneous leishmaniasis disease model was not measured.

Overall, the studies in animal models of visceral and cutaneous disease support activity against *L. donovani, L. infantum, L. mexicana,* and *L. major*.

Drug Resistance

Studies show a potential for development of resistance to miltefosine. Drug resistance could be due to a reduction in drug concentration within the cell. Such a defect was shown to be associated with inactivation or low expression of the miltefosine transport complex in *L. donovani* and *L. braziliensis.* This transporter complex is important for maintaining the integrity of the cell membrane. Some strains of *L. braziliensis* are considered to be intrinsically resistant to miltefosine due to a low expression of miltefosine transporter and protein complex. Overexpression of ABC transporters located in the plasma membrane of *L. infantum* and *L. tropica* and changes in the length and level of unsaturation of fatty acids, as well as a reduction in ergosterol levels suggest that fatty-acid and sterol metabolism are probably also targets for miltefosine resistance.

Point mutations (e.g., L832F) within the *L. donovani* miltefosine transporter locus, observed in one patient with visceral leishmaniasis, may be responsible for the resistant phenotype through inactivation of the protein.

Some strains of *L. braziliensis* are considered to be intrinsically resistant to miltefosine due to low expression of transporter protein complex.

No cross-resistance between miltefosine and drugs currently available in the world for the treatment of leishmaniasis were included in the submission or available by an independent literature search.

Clinical Microbiology

The clinical microbiology measurements in the clinical studies included parasitological diagnosis at baseline based on smear/aspirate by microscopy; at follow-up visits, specimens for parasitological measurements were collected if clinically indicated.

Different *Leishmania* species are morphologically indistinguishable. Species may be differentiated by isoenzyme analysis, molecular methods such as polymerase chain reaction, or fluorescent antibody tests using monoclonal antibodies; however, these assays are not FDA cleared tests. In some of the studies (cutaneous leishmaniasis and mucosal leishmaniasis), efforts were made to identify the *Leishmania* species by one of these tests for research purposes. However, the details of the methods and performance characteristics of the assays in the laboratories where testing was performed were not available for an independent review. Therefore, the results and discussion of the clinical trial findings (cure rates) in this review were mainly based on *Leishmania* species known to be prevalent based epidemiological findings in different geographic regions and not by *Leishmania* species identified by experimental methods (Table 2).

Table 2: Summary of *Leishmania* species in different geographic areas based on epidemiology and experimental methods

Study (Region)	Species* ! (Prevalence)	Species identification in the clinical trials	
		Method	Species identified**
Viseral leishmaniasis			
3154 (India)	*L. (L.) donovani* (100%)	ND	ND
Z025 (Ethiopia)	*L. (L.) donovani* (100%)	ND	ND
033 (India)	*L. (L.) donovani* (100%)	ND	ND
3089 (India)	*L. (L.) donovani* (100%)	ND	ND
3109 (India)	*L. (l.) donovani* (100%)	ND	ND
3127 (India)	*L. (L.) donovani* (100%)	ND	ND
3091 (India)	*L. (L.) donovani* (100%)	ND	ND
3206 (India)	*L. (L.) donovani* (100%)	ND	ND
Z013 (India, Nepal)	*L. (L.) donovani* (100%)	ND	ND
Rahman *et al*., 2009 (Bangladesh)	*L. (L.) donovani* (100%)	ND	ND
Z019 (Brazil)	*L. (L.) chagasi*	ND	ND
CSRI (Europe Spain, France, Italy, Portugal)	*L. (L.) infantum*	ND	ND
Mucocutaneous leishmaniasis			
Z022 (Bolivia)	*L. (V.) braziliensis* (85%)	Skin test/Isoenzyme (n=8)	*L. (V.) braziliensis*
Cutaneous leishmaniasis			
3168 (South America 2 sites):			
Columbia[1]	*L. (V.) panamensis* (54%) *L. (V.) braziliensis* (30%) *L. (L) chagasi* (9%) *L. (V.) guyanensis* (3%) *L. (L.) amazonensis* (1.8%)	IFA (monoclonal antibody used identifies *Leishmania* genus and not the species)	Assay does not identify *Leishmania* species
Guatemala	*L. (V.) braziliensis* (67%) *L. (L) mexicana* (33%)	PCR	*L. (V.) braziliensis* (n=16), *L. (L.) mexicana* (n=14)
Z020a (Manaus, Brazil)	*L. (V.) guyanensis* (99%)	PCR	*L. (V.) guyanensis* (n=58) *L. (V.) lainsoni* (n=1) *L. (V.) braziliensis* (n=1)
Z020b (Bahia State, Brazil)	*L. (V.) braziliensis* (96%) *L. (L.) amazonensis*	PCR / Skin test (*L. amazonensis* antigen)	Identification based on genus Leishmania and subgenus *Viannia*. Therefore, presumed to be *L. (V.) braziliensis* in all patients
Soto (Bolivia)	*L. (V.) braziliensis* (85%)	ND	ND
3092 (Colombia)	see above for Study 3168	Isoenzyme	*L. (V.) panamensis* (n=10) *L. (L.) amazonensis* (n=5)
Z026 (Kabul, Afghanistan)	*L. (L.) tropica*	ND	ND
Diffuse cutaneous leishmaniasis			
Z027 (Venezuela)	*L. (L.) amazonensis* *L. (L.) mexicana*	Isoenzyme and sequencing	*L. (L.) amazonensis* (n=11) *L. (L.) mexicana* (n=2)

**Leishmania* species based on epidemiological findings Old World and New World ;
! Enrollment based on clinical + parasitology by microscopy
***Leishmania* species as specified by the applicant or authors of the publication; Details of the methods and performance characteristics not available for review
Text in red represents pivotal studies
N represents number of patients in the miltefosine treated group; ND-not done

[1] Corredor A, Kreutzer RD, Tesh RB, Boshell J, Palau T, Caceres E, Duque S, Pelaez D, Rodriguez G, Nichols S, Hernandez CA, Morales A, Young DG, and de Carrasquilla CF. Distribution and etiology of leishmaniasis in Colombia. Am J Trop Med Hyg (1990) 42 (3): 206-214.

- **Visceral leishmaniasis**

The efficacy and safety of miltefosine was measured in patients with visceral leishmaniasis in one pivotal study (Study 3154) in India, and a supportive study (Study Z025) in Ethiopia. Additionally, other study reports available for review included dose ranging studies in India, phase 4 studies in the Indian subcontinent conducted after registration of the drug in India, and compassionate use in Europe (Spain, France, Italy, and Portugal). Parasitological diagnosis at baseline was based on smear/aspirate by microscopy. Parasitological observations at the follow-up visits were performed if clinically indicated. No efforts were made to identify *Leishmania* species. However, based on epidemiologic findings, *L. donovani* is known to be the etiological agent for visceral leishmaniasis in India, Bangladesh, and Nepal, *L. chagasi* in Brazil and *L. infantum* in Europe (Table 2).

Miltefosine was effective for the treatment of visceral leishmaniasis. Cure rates in miltefosine treated subjects were similar to the comparator arm (amphotericin B in Study 3154 and sodium stibogluconate in Study Z025). The cure rates were higher in Study 3154 conducted in India compared to Study Z025 in Ethiopia. Such differences in cure rates could be due to differences in protocol design, patient population, severity of disease in Ethiopia compared to India, or strain differences. The mortality rates, measured in Study Z025, were lower in miltefosine treated subjects compared to the control group. In Study Z025, the HIV sero-status was measured. The cure rates were lower and relapse rates higher in HIV^+ subjects compared to HIV^- subjects. Relapse rates were higher in miltefosine treated subjects in both studies compared to the comparators suggesting greater possibility of resistance to miltefosine compared to amphotericin B or sodium stibogluconate.

Additional studies (dose finding and phase 4 studies in India, Bangladesh, and Nepal) also support efficacy of miltefosine for the treatment of visceral leishmaniasis in areas known to be endemic for *L. donovani*. A study in Brazil and a compassionate use of miltefosine in Europe support efficacy of miltefosine for the treatment of visceral leishmaniasis in areas known to be endemic for *L. chagasi* and *L. infantum*, respectively.

- **Mucosal leishmaniasis**

The efficacy and safety of miltefosine was measured in patients with mucosal leishmaniasis in one study (Study Z022) in Bolivia. Culture results were available from 8 patients and all were stated to be *L. braziliensis* by isoenzyme analysis (Table 2). About 85% of Bolivian leishmaniasis is known to be caused by *L. braziliensis.* Mucosal scores decreased by approximately 60% by 2 months post-treatment and 65% of the 76 subjects in the per protocol population were "cured" by the definition of $\geq$ 90% reduction in the mucosal severity score at the 12-month follow-up. The severity score prior to treatment appeared to have an effect on response to treatment. Patients that were cured had a lower severity score at baseline compared to those with a higher severity score and there was a trend toward lack of cure for distal disease versus solely proximal disease. However, there was no correlation between parasite-positivity at baseline and clinical response.

- **Cutaneous leishmaniasis**

The efficacy and safety of miltefosine was measured in patients with cutaneous leishmaniasis in one pivotal study (Study 3168 in Guatemala and Colombia) and 5 supportive studies [Studies Z020a (Manaus, Brazil) and Z020b (Bahia, Brazil), Study Soto (Bolivia), Study 3092 (Colombia), and Study Z026 (Kabul, Afghanistan)]. All the studies except one study (Study

Z026 in Kabul, Afghanistan) were conducted in South America (Colombia and Guatemala, Brazil, or Bolivia). Attempts were made to identify *Leishmania* species in some of the patients in 4 studies (Study 3168, Z020a, Z020b, and 3092) by indirect fluorescent assay (IFA), polymerase chain reaction, or isoenzyme analysis (Table 2). However, these are experimental assays and were performed by the Investigators for research purposes. The details of the methods and performance characteristics of the assays in the laboratories where testing was performed were insufficient for an independent review. It is noteworthy that the references provided for the IFA test used in Study 3168 in Colombia, support the identification of *Leishmania* genus but not *Leishmania* species as the monoclonal antibody used in the test recognizes a dominant antigen common to promastigotes of isolates from 3 major species and 5 subspecies of New World cutaneous leishmaniasis. Therefore, the assay used is not useful for identification of any *Leishmania* species. In the absence of review of details of the methods and performance characteristics of the assays, the results were analyzed by region and not *Leishmania* species reported in some of the studies.

Follow-up for parasitological response was performed if clinically indicated. Overall, the studies suggest miltefosine to be effective in the treatment of cutaneous leishmaniasis in areas known to be endemic for *L. panamensis, L. guyanensis, L. mexicana, L. braziliensis,* and *L. amazonensis* in the New World and *L. tropica* in Old World (Table 2). However, the cure rates may vary in different regions even against the same *Leishmania* species. A comparison of the cure rates for miltefosine against *Leishmania* species in Brazil (Bahia State, Study Z020b – 60%), Bolivia (Study Soto – 72%), and Colombia (Study 3168 - 85%) with that in Guatemala (Study 3168 – 50%) suggests that efficacy in one endemic region cannot be relied on to pertain to other endemic regions, even if the apparently "same" species of *Leishmania* is present in both regions (Table 2). This could be due to differences in the prevalence of *Leishmania* species and their sensitivity to the drug. A decrease in response rate due to acquired or intrinsic resistance to miltefosine cannot be ruled out.

Diffuse cutaneous leishmaniasis
Miltefosine treatment was effective in severely affected diffuse cutaneous leishmaniasis patients in Venezuela known to be endemic for *L. amazonensis* and *L. mexicana*. While complete healing and negative parasitology of skin lesions was achieved after treatment durations ranging from 8-12 weeks relapses were observed in nearly all patients suggesting development of resistance.

2. INTRODUCTION AND BACKGROUND

The subject of this submission is miltefosine (Impavido®) for the treatment of adolescent and adult patients with visceral, mucosal, and cutaneous leishmaniasis. In the United States, intravenously administered liposomal amphotericin B (AmBisome) is approved for the treatment of visceral leishmaniasis. There are no FDA approved therapies for mucosal leishmaniasis or cutaneous leishmaniasis. The applicant was granted a priority review of this application.

2.1. Miltefosine

Miltefosine (hexadecylphosphocholine) is an alkyl phospholipid analog that was originally developed as an anti-neoplastic drug. Miltefosine is currently licensed in Germany, several countries in South East Asia as well as Central and South America for the treatment of visceral leishmaniasis. Miltefosine is approved for treatment of cutaneous leishmaniasis and visceral leishmaniasis in Germany, Colombia, and India.

Miltefosine is homologous to endogenous alkyl-phospholipids that are known to be present in abundance in *Leishmania* spp.

2.2. Biology of *Leishmania*

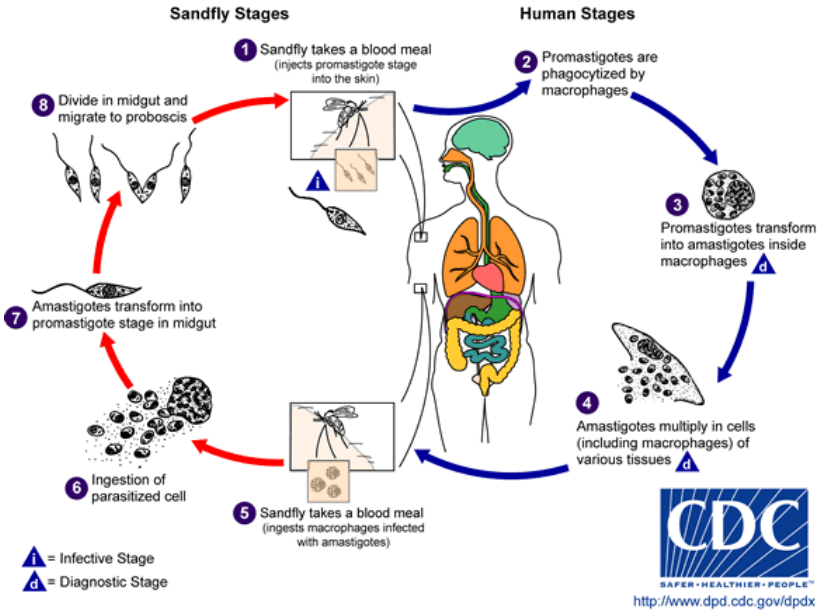
Leishmaniasis is caused by obligate intracellular protozoa of the genus *Leishmania* and is a vector-borne disease transmitted by sandflies. Only female sandflies feed on blood. The disease is a zoonotic disease; the reservoir hosts include rodents and dogs. The disease may also be spread anthroponotically. The sandfly injects the flagellated promastigotes, during the blood meal, into the host's skin. Promastigotes are deposited on the skin into a small pool of blood drawn by the probing sandfly. The organisms fix complement onto their surface by the alternative pathway and are rapidly phagocytozed by host macrophages via the type 3 complement receptor (CR3) and the mannosyl/fucosyl receptor. Within the phagolysosome of a macrophage, the promastigotes(s) transform into non-flagellated amastigotes (Figure 1). The amastigotes replicate by binary fission leading to rupture of the infected cell and infection of other reticulo-endothelial cells. When a sandfly bites an infected host, the ingested amastigotes transform back to promastigotes and develop in gut and migrate to the proboscis.

Essential nutrients, cations and carbon sources are delivered to the phagolysosome via the endocytic pathway or directly from the macrophage cytosol (Figure 1). Amastigotes might internalize low molecular weight nutrients (such as hexose, amino acids, polyamines, purines and vitamins) and cations (Fe^{2+}, Mn^{2+}) via plasma membrane transporters, often in competition with phagolysosome membrane transporters. They also internalize large macromolecules (such as proteins, carbohydrates, DNA and RNA by endocytosis. Heme can be obtained by endocytosis of host proteins or through uptake of free heme by flagellar pocket (FP) receptors. A tight junction might form between the posterior membrane of the amastigote and the phagolysosome membrane, and be involved in scavenging host lipids.

Human infection is caused by about 21 of 30 species that infect mammals and are classified in two subgenera: *Leishmania* and *Viannia* (Figure 2). The subgenus *Leishmania* include the *L. donovani* complex with 2 species (*L. donovani, L. infantum* [also known as *L. chagasi* in the New World)], the *L. mexicana* complex with 3 main species *(L. mexicana, L. amazonensis,* and *L. venezuelensis), L. tropica, L. major,* and *L. aethiopica;* the subgenus *Viannia* includes 4 main species *(L. braziliensis, L. guyanensis, L. panamensis,* and *L. peruviana).* The different *Leishmania* species are morphologically indistinguishable.

Figure 1: Life cycle of *Leishmania* species

Life cycle



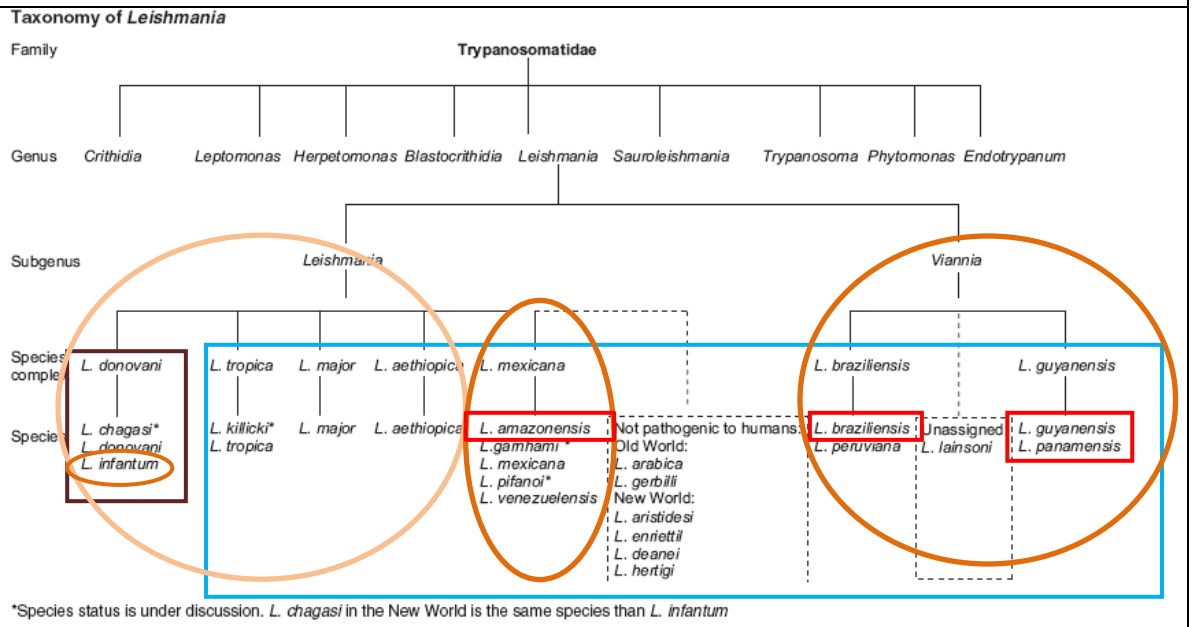
Leishmaniasis is transmitted by the bite of infected female phlebotomine sandflies. The sandflies inject the infective stage (i.e., promastigotes) from their proboscis during blood meals 1. Promastigotes that reach the puncture wound are phagocytized by macrophages 2 and other types of mononuclear phagocytic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

Source: http://www.cdc.gov/parasites/leishmaniasis/biology html

Life of an amastigote in the phagolysosome

Source: McConville MJ, de Souza D, Saunders E, Likic VA, and Naderer T. Living in a phagolysosome; metabolism of *Leishmania* amastigotes. Trends in Parasitology (2007) 23 (8):368-375.

Figure 2: Taxonomy of Leishmaniasis and geographic distribution



Adapted from Control of the leishmaniasis: Report of a meeting of the WHO Expert Committee on the Control of Leishmaniasis, Geneva, 22-26 March 2010 - http://www.who.int/leishmaniasis/en/

Visceral leishmaniasis; Mucocutaneous leishmaniasis; Cutaneous leishmaniasis

Old World *Leishmania* species; New World *Leishmania* species

Parasite	Locality
Subgenus *Leishmania* (Ross, 1903)	
L. donovani phenetic complex	
L. donovani (Laveran and Mesnil, 1903)	India, China, Bangladesh
L. archibaldi (Castellani and Chalmers, 1919)	Sudan, Ethiopia
L. infantum phenetic complex	
L. infantum (Nicolle, 1908)	North central Asia, northwest China, Middle East, southern Europe, northwest Africa
L. chagasi (Cunha and Chagas, 1937)	South and Central America
L. tropica phenetic complex	
L. tropica (Wright, 1903)	Urban areas of Middle East and India
L. killicki (Rioux, Lanotte, and Pratlong, 1986)	Tunisia
L. major phenetic complex	
L. major	Africa, Middle East, Soviet Asia
L. gerbilli phenetic complex	
L. gerbilli (Wang, Qu, and Guan, 1973)	China, Mongolia
L. arabica phenetic complex	
L. arabica (Peters, Elbihari, and Evans, 1986)	Saudi Arabia
L. aethiopica phenetic complex	
L. aethiopica (Bray, Ashford, and Bray, 1973)	Ethiopia, Kenya
L. mexicana phenetic complex	
L. mexicana (Biagi, 1953)	Mexico, Belize, Guatemala, South central United States
L. amazonensis (Lainson and Shaw, 1972)	Amazon Basin, Brazil
L. venezuelensis (Bonfante-Garrido, 1980)	Venezuela
L. enrietti phenetic complex	
L. enrietti (Muniz and Medina, 1948)	Brazil
L. hertigi phenetic complex	
L. hertigi (Herrer, 1971)	Panama, Costa Rica
L. deanei (Lainson and Shaw, 1977)	Brazil
Subgenus *Viannia* (Lainson and Shaw, 1987)	
L. braziliensis phenetic complex	
L. braziliensis (Viannia, 1911)	Brazil
L. peruviana (Velez, 1913)	Western Andes
L. guyanensis phenetic complex	
L. guyanensis (Floch, 1954)	French Guiana, Guyana, Surinam
L. panamensis (Lainson and Shaw, 1972)	Panama, Costa Rica

In other classifications, subspecies of *L. mexicana* have been recognized, and these names—*L. mexicana aristedesi*, *L. m. garnhami*, and *L. m. pifanoi*—do appear in the literature, with the subspecific name sometimes used as a specific epithet. The groupings in the table are those of Rioux et al.[89] and are based on extensive isozyme and cladistic analysis. The term *phenetic complex* refers to zymodemes revealed by cluster analysis.

Source: http://dna.kdna.ucla.edu/parasite_course-old/leish_files/introduction1.htm

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About 350 million people are considered at risk of contracting leishmaniasis[2] in tropical and sub-tropical regions. *Leishmania* infections that occur in Asia, Africa, Europe and the Middle East are designated as Old World, while infections that occur in the Americas are designated as New World. *L. panamensis, L. braziliensis, L. guyanensis, L. mexicana, L. major,* and *L. tropica* account for most infections in U.S. travelers or military populations.

Pathogenesis
The clinical outcome of infection depends on the host's immune response, the characteristics of the infecting *Leishmania* species, and endemic region. In general, clinical syndromes are divided into cutaneous leishmaniasis, mucosal leishmaniasis and visceral leishmaniasis. **Cutaneous** leishmaniasis is the most common form of infection and characterized by skin lesions and may self-heal without treatment. When infections metastasize to the mucous membrane (**mucosal** or **mucocutaneous** leishmaniasis) or internal organs such as liver and spleen (**visceral** leishmaniasis), the infection may be fatal without treatment. Mucosal disease is due to infection with some New World species, whereas cutaneous and visceral disease can be due to infections with New or Old World species. **Diffuse cutaneous leishmaniasis**, although rare, is profoundly incapacitating and shares features with visceral leishmaniasis, including a defect in specific cell-mediated immune responses during active disease and a heavy parasitic burden.

Cutaneous infections are caused by 20 *Leishmania* species which are referred to as Old World (Asia, Africa and the Middle East) or New World (Americas) species (Figure 2); the number of lesions and the size of lesions may vary.

Mucosal leishmaniasis is caused typically by *L. braziliensis* and rarely by *L. amazonensis, L. guyanensis*, and *L. panamensis*.

Visceral leishmaniasis is caused by the *L. donovani* complex, which includes *L. donovani* and *L. infantum* (the latter designated as *L. chagasi* in the New World); it is the most serious form of the disease, which is fatal if left untreated.

Diffuse cutaneous leishmaniasis is most frequently caused by *L. amazonensis* in the New World; cases of diffuse cutaneous leishmaniasis produced by *L. braziliensis* have been reported in immunocompromised individuals. In the Old World, the most frequent cause of diffuse cutaneous leishmaniasis is *L. aethiopica*.

Diagnosis
Diagnosis of leishmaniasis is by visualizing amastigotes in smears of tissue aspirates (such as skin, nasal mucosa, lymph nodes, bone marrow and spleen) or skin scrapings or biopsies; this is usually performed by Giemsa staining of smears, culture *in vitro,* or passage in animals (hamsters).

The different *Leishmania* species are morphologically indistinguishable. Species may be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies; however, these assays are not FDA cleared tests.

Leishmania species are thought to vary in both membrane sterol and lipid content; it is possible that the biochemical composition of these parasites might affect drug activity. *Leishmania*

[2] Control of leishmaniasis. WHO technical report series 949 (2010).

parasites have high levels of phospholipid content in plasma membranes (65–80% of the total lipid content) principally phosphatidycholine, phosphatidylethanolamine, phosphatidyinositol and diphosphatidylglycerol.

3. NON CLINICAL (PROOF OF CONCEPT) STUDIES

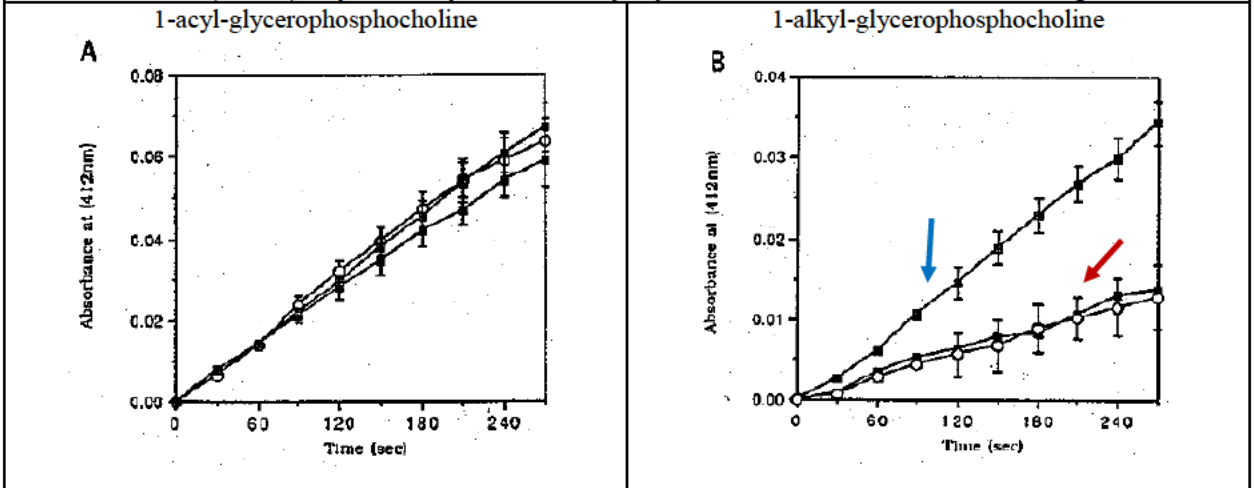
3.1. Mechanism of action

The effect of miltefosine on alkylphospholipid synthesis, phosphocholine transport, lipid monolayer, cytochrome c oxidase (mitochondrial respiratory chain), apoptosis like death, and immune system was investigated.

3.1.1. Effect on alkylphospholipid synthesis

Studies by Lux *et al.* (1996[3] and 2000[4]) reported the effect of miltefosine on the acyl and alkyl phospholipid acyltransferases that are important for alkylphospholipid synthesis in *L. mexicana* promastigotes (MNYC/BZ/62/M379 strain). Inhibition of acyl-CoA transferase enzyme activity, essential for fatty acid synthesis, was measured by spectroscopy at 412 nm. Complete details of experimental design were not available in the publications. The results showed that miltefosine had only moderate inhibitory effect on 1-acyl-glycerophosphocholine:1-acyl-CoA acyltransferase activity (Figure 3A), a key enzyme in acyl glycerolipid metabolism. However, miltefosine inhibited 1-alkyl-glycerophosphocholine:1-acyl-CoA acyltransferase activity by about 60% (Figure 3B) suggesting ether lipid re-modelling enzyme was more sensitive to alkyl phospholipid analogues than the ester phospholipid counterpart. The concentration of miltefosine used for testing was not specified.

Figure 3: Effect of miltefosine on (A) 1-acyl-glycerophosphocholine (2-LPC); and (B), 1-alkyl-glycerophospho-choline (L-PAF): acyl-CoA acyl transferase by acylation reactions for *L. mexicana* homogenates



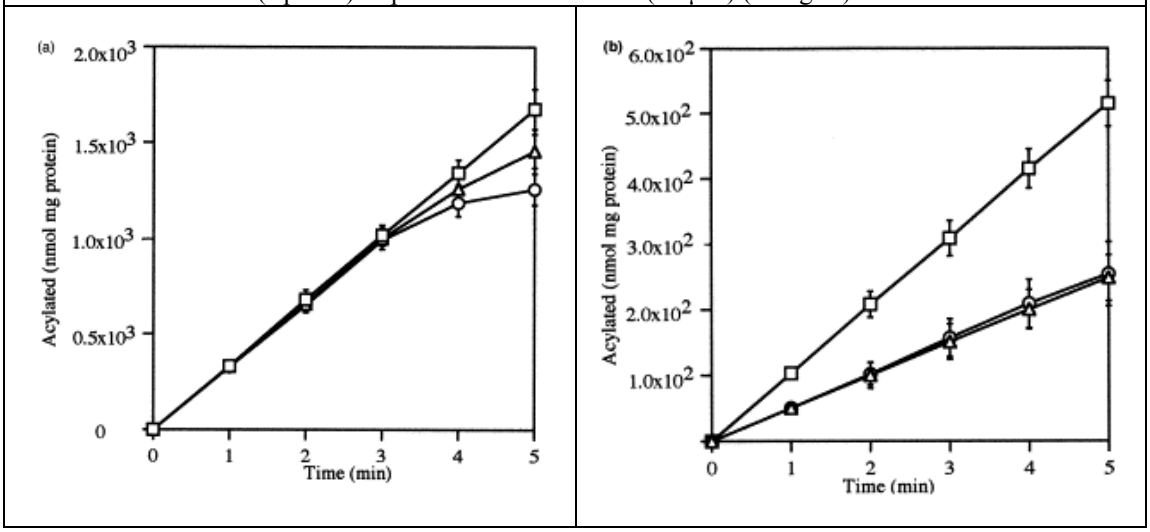
Blue arrow represents control. Red arrow represents miltefosine

[3]Lux H, Hart DT, Parker PJ, and Klenner T. Ether lipid metabolism, GPI anchor biosynthesis, and signal transduction are putative targets for anti-leishmanial alkyl phospholipid analogues. Adv Exp Med Biol (1996) 416: 201–211.

[4] Lux H, Heise N, Klenner T, Hart D, and Opperdoes F R. Ether-lipid (alkyl-phospholipid) metabolism and the mechanism of action of ether-lipid analogues in *Leishmania*. Mol Biochem Parasitol (2000) 111:1–14.

In another experiment, the effect of miltefosine (50 µM i.e., 20.4 µg/mL) on acyl- and alkyl-*lyso*-phosphatidyl choline (PtdCho) acyltransferases was measured in glycosome-enriched fractions from promastigotes of *L. mexicana*. The results showed that the alkyl-*lyso*-PtdCho acyltransferase product, alkyl-lyso-PtdCho, is reduced 2-3 fold in the presence of miltefosine while the acyl-*lyso*-PtdCho product, acyl-*lyso*-PtdCho, is not substantially affected (Figure 4); such an effect was at high concentrations of miltefosine (50 µM i.e., 20.4 µg/mL).

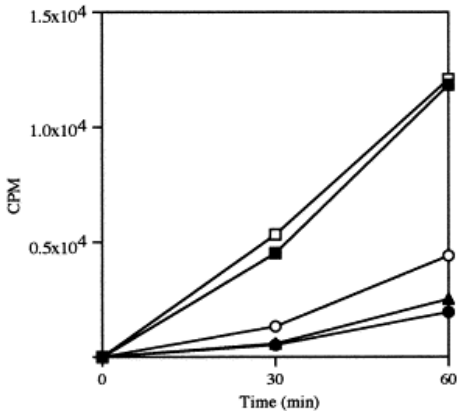
Figure 4: The effect of miltefosine on acyl- and alkyl-*lyso*-phosphatidylcholine acyltransferase activity in glycosome-enriched fractions from *L. mexicana.* (a) Acyl-*lyso*-PtdCho acyltransferase and (b) alkyl-*lyso*-PtdCho acyltransferase activities were measured using oleoyl-CoA (50 µM) in the absence (squares) or presence of miltefosine (50 µM) (triangles).



The involvement of glycosomes in possible remodeling pathways was further confirmed using radiolabelled substrates to identify the corresponding PtdCho acyltransferase products. The results showed that highly purified glycosomes were capable of forming diacylPtdCho and alkyl-acyl-PtdCho. Diacyl-PtdCho production lacked sensitivity to miltefosine; miltefosine inhibited the formation of the alkyl-acylPtdCho counterpart (Figure 5). Dose dependent inhibition of glycosomal alkyl-lyso-PtdCho metabolism was observed over a 10-fold substrate concentration.

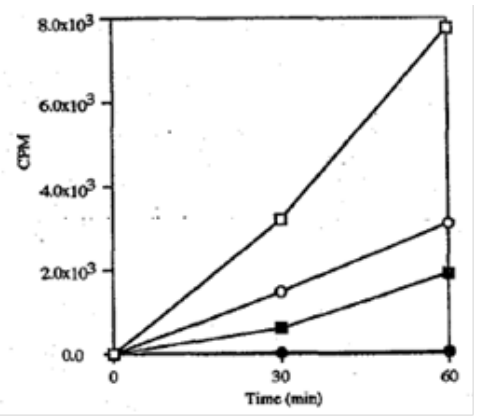
Figure 5: The effect of miltefosine on the production *in vitro* of [1-^{14}C] palmitic acid-labeled by purified glycosomes from *L. mexicana* promastigotes. (A) phosphatidylcholine and (B) alkyl-acyl-phosphatidylcholine

(A)



Acyl-*lyso*-PtdCho and alkyl-*lyso*-PtdCho acyl-transferase activities were measured radio-chemically using 100 µM of either acyl-*lyso*-PtdCho (open squares) or alkyl-*lyso*-PtdCho (open circles) as substrates in the absence or presence of miltefosine (100 µM) (closed squares and circles).

(B)



The acyltransferase activities were measured radiochemically using to 10 µM (open circles) or 100 µM (open squares) of alkyl-lysoPtdCho as substrate and in the presence of 10 µM (closed circles) or 100 µM (closed squares) of miltefosine, respectively.

Overall, the results showed that miltefosine inhibited acyl-CoA transferase activity and perturbed the later steps of alkyl-phospholipid biosynthesis. The authors state that it is unclear if this pathway is the primary target as such changes were observed at high miltefosine concentration (50 µM i.e., 20.4 µg/mL).

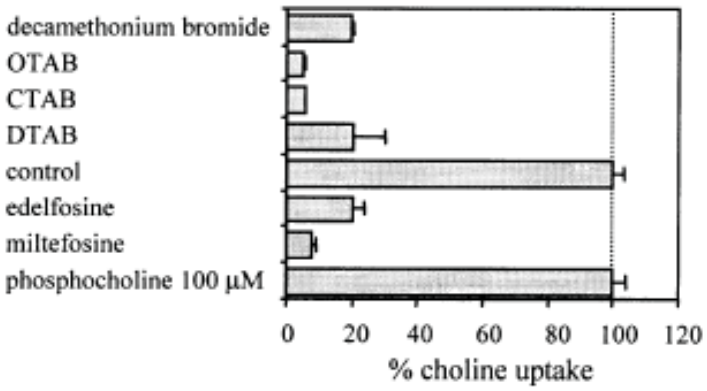
3.1.2. Effect on choline transport

In a study by Zufferey and Mamoun (2002)[5] the effect of miltefosine on choline uptake by *L. major* promastigotes of Friedlin strain VI (MHOM/IL/80/Friedlin) was reported. Phosphatidylcholine is the major phospholipid in the *Leishmania* membranes, representing 33% of total lipids and 50% of phospholipids in this parasite. Choline has been postulated to play an essential role in *Leishmania* development, implying that the parasite has the enzymatic machinery to synthesize phosphatidylcholine *de novo* from choline, and requires a transporter for uptake of choline from the host.

Promastigotes (about 10^7 cells) were used for the choline uptake assay using 1 mCi of radiolabeled ^{3}H-methylcholine in the absence or presence of various concentrations of cold choline or inhibitors including miltefosine. The mixture was incubated at 0 or 26^{o}C, for 3.5 minutes, and the reaction terminated by filtering the cell suspension through GF/C (Whatman) membranes that were then washed with cold PBS; filters were dried, mixed with scintillation fluid and counted. The results showed 10 µM i.e., 4.1 µg/mL miltefosine inhibited the uptake of choline to 8% of the control level (Figure 6). The study suggests that miltefosine may use the choline transporter(s) to cross the plasma membrane of the *L. major* parasite and that inhibition of choline transport may contribute to the mechanism of action of the drug.

[5] Zufferey R and Mamoun CB. Choline transport in *Leishmania* major promastigotes and its inhibition by choline and phosphocholine analogs. Molecular and Biochemical parasitology (2002) 125: 127-134.

Figure 6: Inhibition of choline uptake by choline and phosphocholine analogs at 10 µM; phosphocholine was used at 100 µM.



Choline uptake assay was performed at 26°C for 3.5 min. Each bar represents the average and standard deviation of two measurements. Hundred percent activity corresponds to 4.3 pmol X min^{-1} X 10^7 cells of [^{3}H] methylcholine uptake.

In studies by Rakotomanga *et al*. (2005 and 2007),[6, 7] the inhibitory effect of miltefosine on fatty acid and sterol metabolism of promastigotes of a *L. donovani* strain was reported.

3.1.3. Interaction with lipid monolayers

A study by Rakotomanga *et al*. (2004),[8] reported interaction of miltefosine with lipid monolayers *in vitro*. In this study, the interaction of miltefosine with a monolayer of h-palmitoyl-g-oleyl-phosphatidylcholine (POPC) as membrane model or sterol (ergosterol or cholesterol) was investigated. At a constant pressure (25 mN/m), miltefosine molecules were inserted into the monolayer of lipids as monomers until the critical micellar concentration (CMC). At higher miltefosine concentrations, the micelles of miltefosine were deployed at the interface as groups of monomers into the POPC or sterol monolayer. The study of mixture of miltefosine/POPC or sterol, spread at the air–water interface, showed a simple miscibility between miltefosine and POPC, whereas a high condensation appeared between miltefosine and sterols suggesting high affinity for sterols. Miltefosine did not act as detergent disturbing membrane integrity.

3.1.4. Effect on cytochrome c oxidase/mitochondria

The involvement of mitochondrial dysfunction was reported in *L. panamensis* promastigotes; the mitochondrial membrane potential was substantially reduced after incubation with miltefosine.[9]

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[6] Rakotomanga M, Saint-Pierre-Chazalet M, and Loiseau PM. Alteration of fatty acid and sterol metabolism in miltefosine-resistant *Leishmania donovani* promastigotes and consequences for drug–membrane interactions. Antimicrob Agents Chemother (2005) 49: 2677–2686.

[7] Rakotomanga M, Blanc S, Gaudin K, Chaminade P, and Loiseau M. Miltefosine affects lipid metabolism in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother (2007) 51:1425–1430.

[8] Rakotomanga M, Loiseau PM, and Saint-Pierre-Chazalet M. Hexadecylphosphocholine interaction with lipid monolayers. Biochimica et Biophysica (2004) 1661: 212-218.

[9] Santa-Rita RM, Henriques-Pons A, Barbosa HS, de Nazareth M, Meirelles SL and de Castro SL. Effect of the lysophospholipid analogues edelfosine, ilmofosine and miltefosine against *Leishmania amazonensis*. J Antimicrob Chemother (2004) 54: 704–710.

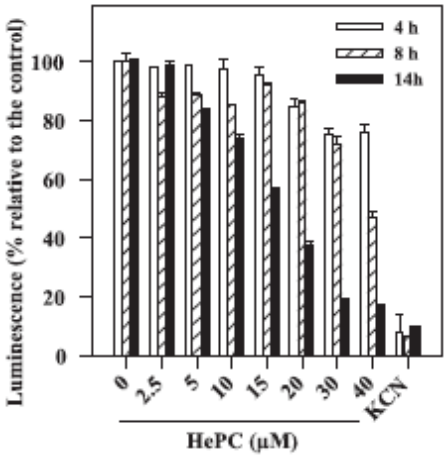
The effect of miltefosine on cytochrome c oxidase within the mitochondria was investigated (Luque-Ortega *et al.*, 2007)[10]. For this, *L. donovani* 3-Luc strain was obtained by transfection of promastigotes of *L. donovani* strain MHOM/SD/00/1S-2D; transfection was performed with the expression vector pX63NEO-3Luc, which encodes a cytoplasmic form of the *Photinus pyralis* luciferase mutated at its C-terminal tripeptide. The *in vivo* luminescence of strain 3-Luc promastigotes affords the real-time monitoring of changes in the cytoplasmic level of ATP. In promastigotes exposed to miltefosine, concentration- and time-dependent decreases in luminescence and the intracellular ATP pool were observed (Figure 7); after incubation with 15 µM i.e., 6.1 µg/mL of miltefosine for 14 hours, the luminescence decreased by half. Such a decrease in ATP pool may reflect an overall deterioration of parasite homeostasis, specific effects of miltefosine on processes directly involved in ATP synthesis or to accelerated ATP decay. The authors state that accelerated ATP decay is not possible due to scarce intracellular accumulation of SYTOX green observed.

In another experiment, oxidative phosphorylation as a plausible target for miltefosine was investigated, since in *Leishmania* this process is known to be the main source of ATP. The results showed a decrease in oxygen consumption (Figure 7). Miltefosine fully inhibited TMPD (tetramethyl-*p*-phenylenediamine)-ascorbate-dependent oxygen consumption, which provides evidence of the specific inhibition of cytochrome c oxidase. This was further corroborated by measuring the inhibition of this enzyme by increasing miltefosine concentrations in mitochondrial fractions. The results showed direct and dose-dependent inhibition of cytochrome c oxidase activity in the presence of miltefosine. Total inhibition of this enzyme was achieved with 10 mM KCN, used as a reference (Figure 7). The specificity of this effect was confirmed by the fact that the activity of cytochrome c reductase (complex III) in the mitochondrial fraction was barely inhibited (5%) with 40 µM i.e., 16.3 µg/mL miltefosine, the highest concentration tested (data not shown).

[10] Luque-Ortega JR and Rivas L. Miltefosine (Hexadecylphosphocholine) inhibits cytochrome *c* oxidase in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother (2007) 51 (4): 1327-1332.

Figure 7: Effect on cytochrome c oxidase *in vitro*

Variation of *in vivo* luminescence of strain 3-Luc *L. donovani* promastigotes incubated with miltefosine.



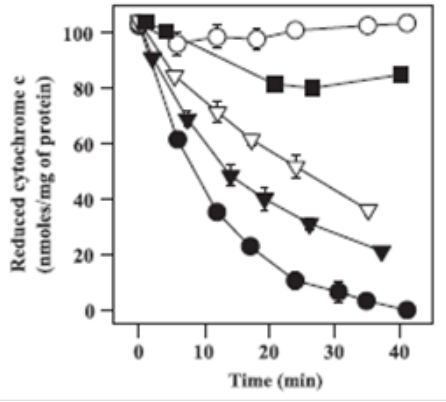
Promastigotes incubated for different times with miltefosine (HePC) at the indicated concentrations were loaded with 25 µM DMNPE-luciferin. The variation in luminescence was normalized relative to that for the untreated parasites. Maximal inhibition of luminescence was achieved with 10 mM KCN.

Effect on oxygen consumption

HePC concn (µM)	O_2 consumption rate (nmol O_2 × min^{-1} × 10^{-8} cells)	% Consumption relative to that for control
0	19.6	100.0
10	18.7	95.4
20	14.7	75.0
40	8.7	44.4

HePC= miltefosine

Effect on cytochrome c oxidase activity



The mean cytochrome c oxidase activity ± SD was monitored by determination of the decrease in the absorbance at 550 nm that a reduced cytochrome *c* solution (32 µM) underwent when it was oxidized by cytochrome c oxidase at 37°C. The spontaneous oxidation rate was determined in samples previously incubated with 10 mM KCN. The following miltefosine concentrations (µM) were tested: 40 (■), 25 (▽), 15 (▼), and 0 (no treatment) (●). ○, 10 mM KCN.

3.1.5. Effect on apoptosis

In a study by Paris *et al*. (2004),[11] promastigote forms of wild-type (WT) *L. donovani* (strain MHOM/ET/67/HU3/L82) and a derivative line, which is resistant to 40 µM (16.3 µg/mL)

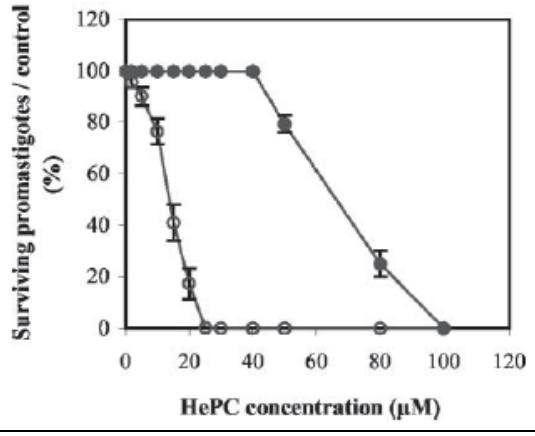
[11] Paris C, Loiseau PM, Bories C, and Breard J. Miltefosine Induces Apoptosis-Like Death in *Leishmania donovani* Promastigotes. Antimicrob Agents Chemother (2004) 852-859.

miltefosine (designated HePC-R40) were included for testing; cultures of the resistant strain were maintained in medium containing 40 µM i.e., 16.3 µg/mL miltefosine.

Analysis of drug sensitivity by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Promastigotes, harvested in the exponential growth phase, were resuspended in fresh medium to achieve 10^6 parasites/mL and seeded in 96-well culture plates. Miltefosine was added in triplicate at final dilutions ranging from 1 to 100 µM i.e., 0.4 to 40.8 µg/mL and cultures incubated at 26°C for 72 hours in a 5% CO_2 atmosphere. Promastigote viability was evaluated by the quantitative colorimetric MTT assay; the conversion of MTT to the formazan product by the mitochondrial electron transport chain is an indicator of cell viability, and a decrease in the amount of MTT converted indicates toxicity to the cell. Briefly, the MTT labeling reagent was added to each well and cultures incubated for additional 4 hours at 26°C; DMSO was added to dissolve the formazan crystals and the absorbance measured with an enzyme-linked immunosorbent assay plate reader (wavelength, 540 nm). The percentage of surviving promastigotes versus the number of surviving control promastigotes was assessed by the formula 100 X (absorbance of treated cells/absorbance of control cells). The 50% inhibitory concentration (IC_{50}), i.e., the drug concentration that decreases the rate of cell growth by 50%, was calculated by regression analysis; and the results were expressed as the means and standard deviations of five independent experiments.

Miltefosine decreased the viability of promastigotes in a concentration-dependent manner (Figure 8), with an IC_{50} of 13.6 ± 2.0 µM i.e., 5.54 ± 0.8 µg/mL. However, miltefosine at concentrations up to 40 µM i.e., 16.3 µg/mL had no cytotoxic effect on clone HePC-R40. This resistance, however, was not absolute, since concentrations of miltefosine higher than 40 µM i.e., 16.3 µg/mL in which this clone is regularly maintained were found to be cytotoxic with an IC_{50} of 69.1 ± 1.1 µM i.e., 28.2 ± 0.45 µg/mL.

Figure 8: Cytotoxic activity of miltefosine on *L. donovani* promastigotes of WT (open circles) and HePC-R40 (closed circles) strains. Data are presented as means of five independent experiments performed in triplicate, and standard deviations are represented by error bars.

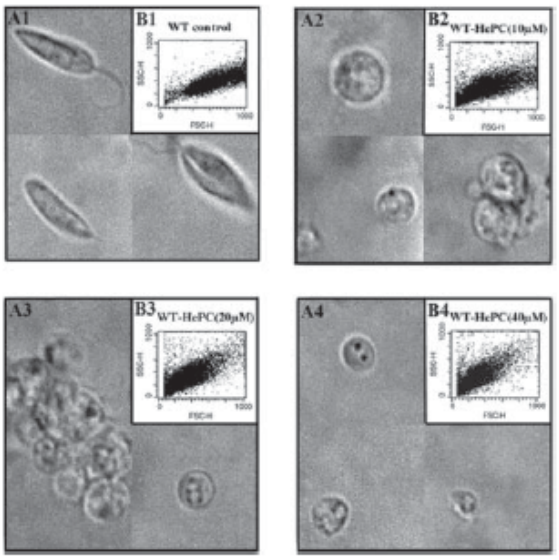


HePC=miltefosine

Miltefosine induced morphological changes in WT promastigotes compared to the morphology of the untreated control group as observed by light microscopy. WT promastigotes cultured for 24 hours in fresh complete medium had elongated forms. Cells exposed to 10 µM (4.1 µg/mL), 20 µM (8.2 µg/mL), or 40 µM (16.3 µg/mL) miltefosine for 24 hours showed rounded forms and cell shrinkage, particularly at the higher dose. Moreover, flow cytometry analysis of cell size

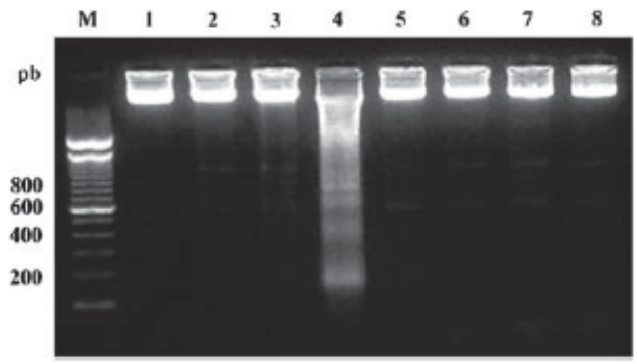
confirmed that miltefosine caused cell shrinkage in WT promastigotes, measured by the decrease in forward scatter, which was more pronounced at higher concentrations of miltefosine (Figure 9). No rounding or shrinkage was observed in the resistant clone exposed to similar concentrations of miltefosine.

Figure 9: Morphology and cell size of *L. donovani* WT promastigotes cultured for 24 hours in fresh complete medium at 26°C in the absence or presence of different concentrations of miltefosine. The results of light microscopy (magnification, X 1,000) (A) and flow cytometry analysis (B) of the cell sizes of WT promastigotes either untreated (A1 and B1) or exposed to 10 µM (A2 and B2), 20 µM (A3 and B3), or 40 µM miltefosine (A4 and B4) are shown.



DNA analysis by agarose gel electrophoresis revealed DNA fragmentation into oligonucleosome-sized fragments (in multiples of 200 bp) in WT promastigotes incubated with 40 µM i.e., 16.3 µg/mL miltefosine for 24 hours (Figure 10, lane 4) as compared to drug free control (Figure 10, lane 1) or lower concentrations of miltefosine (Figure 10, lanes 2 and 3, respectively). No DNA fragmentation was detected in clone HePC-R40, in the absence or presence of miltefosine at 20, 40, or 80 µM (8.2, 16.3, or 32.6 µg/mL, respectively) concentration (Figure 10, lanes 5, 6, 7, and 8, respectively). DNA fragmentation of promastigotes in the presence of miltefosine is similar to one of the typical nuclear features of apoptosis in mammalian cells. These observations were confirmed by flow cytometry analysis after cell permeabilization and labeling with propidium iodide (PI); the resistant clone incubated with a higher concentration (80 µM i.e., 32.6 µg/mL) of miltefosine showed DNA fragmentation in a moderate number of these cells (29% and 39% of pseudohypodiploid cells at 24 hours and 48 hours, respectively).

Figure 10: DNA fragmentation analysis by agarose gel electrophoresis.

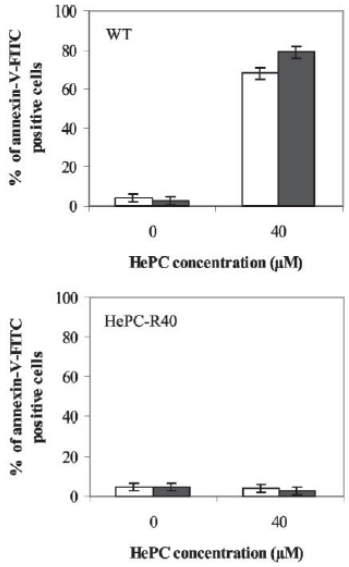


The DNA profiles for untreated or miltefosine-treated *L. donovani* promastigotes after 24 hours of incubation at 26°C are shown. Lane M, molecular size marker (pb, base pairs). WT promastigotes were untreated (lane 1) or were exposed to 10 µM (lane 2), 20 µM (lane 3), or 40 µM miltefosine (lane 4); HePC-R40 promastigotes were untreated (lane 5) or were exposed to 20 µM (lane 6), 40 µM (lane 7), or 80µM miltefosine (lane 8). The results are representative of those from three independent experiments.

In another experiment, the apoptotic effect of miltefosine was evaluated by the use of annexin V, a Ca^{2+}- dependent phospholipid-binding protein with a high affinity for phosphatidyl-serine that is routinely used in a fluorescein-conjugated form to label externalized phosphatidylserine. Since annexin V-FITC can also label necrotic cells following the loss of membrane integrity, simultaneous addition of propidium iodide (PI), which does not permeate cells with an intact plasma membrane, allows the discrimination between apoptotic cells (annexin V positive, PI negative), necrotic cells (annexin positive, PI positive), and surviving cells (annexin V negative, PI negative). The authors state that observation by fluorescence microscopy of WT promastigotes incubated with 40 µM i.e., 16.3 µg/mL miltefosine for 24 or 48 hours showed rounded, condensed cells that were labeled with annexin V-FITC, whereas untreated cells were negative (data not shown). Miltefosine exposure did not induce necrotic features, even after a prolonged incubation, as all the cells remained negative for PI. No apoptotic or necrotic cells were detected in clone HePC-R40, either control cultures or those incubated with the same concentration of miltefosine for 24 or 48 hours (data not shown).

Flow cytometry analysis after labeling with annexin V-FITC was used to quantify the percentage of cells presenting this apoptotic feature. After 24 hours of incubation of WT promastigotes with 40 µM i.e., 16.3 µg/mL miltefosine, 68% of the cells were annexin V-FITC positive whereas only 4% of the control cells were annexin V-FITC positive (Figure 11); after 48 hours the proportion of annexin V-FITC-positive cells incubated with miltefosine increased to 79%, whereas no change was detected in the control group (3%). In contrast, no induction of phosphatidylserine exposure was observed in HePC-R40 promastigotes treated with the same concentration of miltefosine for 24 or 48 hours (Figure 11). Given the lack of PI labeling observed by fluorescence microscopy, PI was not used in these experiments.

Figure 11: Phosphatidylserine exposure analysis by flow cytometry.

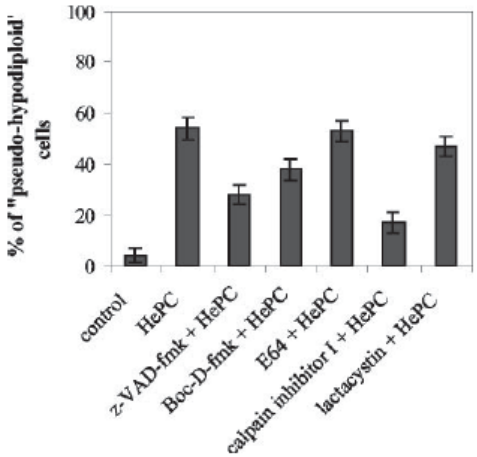


The percentages of annexin V-FITC-positive WT promastigotes (top) or HePC-R40 promastigotes (bottom) were determined after 24 hours (open bars) or 48 hours (closed bars) of incubation at 26°C in the absence or presence of 40 µM miltefosine. The data are the means of three independent experiments, and standard deviations are represented by errors bars.

HePC=miltefosine

In another experiment, efforts were made to evaluate whether proteases could be involved in the induction of apoptosis-like death by miltefosine. For this, two cell-permeant, irreversible, and specific caspase inhibitors (*z*-VAD-fmk and Boc-D-fmk), a specific calpain inhibitor (E64), a broad protease inhibitor (calpain inhibitor I), and an irreversible proteasome inhibitor (lactacystin) were used. Incubation with any of these inhibitors at the concentration used did not cause any cell alteration (data not shown). The authors state that microscopy and flow cytometry analysis showed that none of the inhibitors protected WT promastigotes against cell shrinkage or phosphatidylserine exposure induced by miltefosine (data not shown). Quantitative analysis of pseudohypodiploid cells showed that E64 and lactacystin had a negligible effect; however, the presence of caspase inhibitors reduced the percentage of cells present in the sub-G1 peak region. Such an effect was even more pronounced with calpain inhibitor I (Figure 12).

Figure 12: Effects of protease inhibitors on HePC-induced DNA fragmentation in WT promastigotes.



Parasites were incubated for 2 hours in the presence or absence of the protease inhibitors *z*-VAD-fmk, Boc-D-fmk, or E64 (100 µM), calpain inhibitor I (20 µM), or lactacystin (50 µM) prior to the addition of HePC (40 µM). Promastigotes were incubated for an additional 24 hours at 26°C. The percentage of pseudohypodiploid cells was determined by flow cytometry analysis. The data are the means of three independent experiments, and standard deviations are represented by errors bars.

HePC=miltefosine

Overall, the results showed that miltefosine induced a type of death of *L. donovani* promastigotes WT that shares most of the features associated with metazoan apoptosis i.e., cell shrinkage, DNA oligonucleosomal digestion and phosphadiylserine exposure with preservation of plasma membrane integrity. It also appears that proteases are part of the cell death machinery. However, a clone derived from the same strain by selective pressure and resistant to miltefosine (clone HePC-R40) did not display any apoptosis-like changes when it was exposed to miltefosine concentrations sufficient to kill the WT strain (up to the 40 µM i.e., 16.3 µg/mL used for selection).

In another study (Khademvatan *et al*., 2011[12]), miltefosine was shown to induce programmed apoptosis-like cell death at a concentration equivalent to its IC_{50} values in intra- and extra-cellular stages of *L. infantum*. However, a study by Moreira *et al.* (2011)[13] showed that *Leishmania* mutants resistant to miltefosine, stibogluconate, or amphotericin B not only failed to undergo apoptosis following exposure to their respective drugs.

In another study (Verma and Dey, 2004[14]), apoptosis-like death of *L. donovani* (strain MHOM/80/IN/Dd8) was reported by nuclear DNA condensation, DNA fragmentation with accompanying ladder formation, and *in situ* labeling of DNA fragments by the terminal deoxyribonucleotidyl transferase mediated dUTP-biotin nick end labeling method. The *in vitro* activity against the promastigotes was evaluated by the MTT assay as summarized above except that cultures of promastigotes were incubated with miltefosine for 48 hours. The results showed a slow killing effect up to 20 µM i.e., 8.2 µg/mL concentration of miltefosine and a rapid and dose-dependent cell death at miltefosine concentrations between 30 and 50 µM i.e., 12.2 - 20.4 µg/mL, reaching approximately 100% at around 40 µM (16.3 µg/mL). The IC_{50} value was 25 µM i.e., 10.2 µg/mL (Figure 13). The average number of cells at the time of addition of miltefosine to cultures was 5 x 10^6/mL. The cells exposed to miltefosine grew up to 7 to 8 x 10^6/mL in the next 8 hours after incubation and remained constant till 96 hours compared to the untreated controls, which grew up to 20 to 25 x 10^6/mL till 96 hours. Cell shrinkage after about 4 hours of drug exposure was observed. By the end of 48 hours, almost all the cells showed cytoplasmic condensation and shrinkage, resulting in complete circularization and substantial reduction in size compared to the control samples.

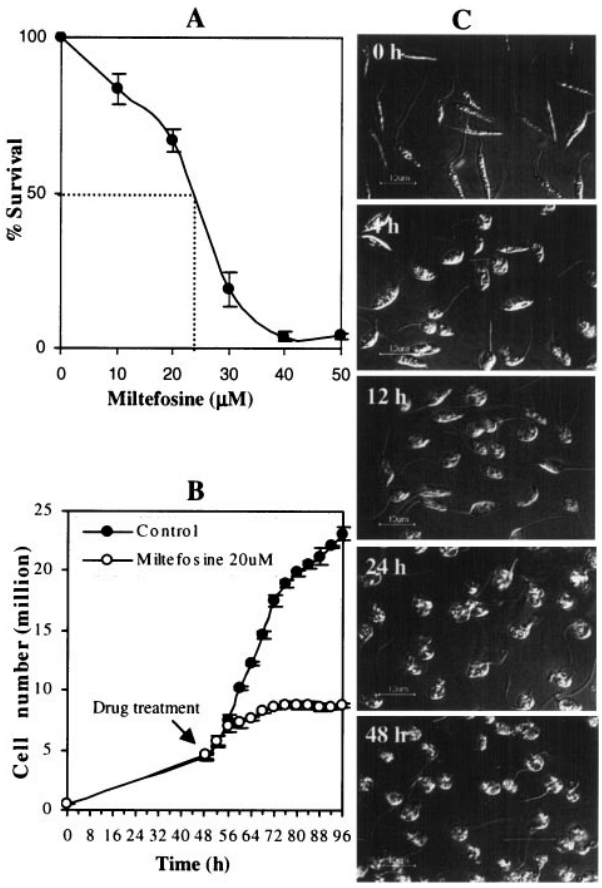
In another experiment, the mode of miltefosine induced death was investigated by the use of PI; condensed nuclei are known to exhibit brighter red fluorescence than noncondensed nuclei, which show dull red fluorescence. Promastigotes incubated with 25 µM (10.2 µg/mL) miltefosine showed bright red fluorescent spots compared to the normal dull red fluorescence in untreated cells suggesting nuclear condensation occurred in *L. donovani* promastigotes during the miltefosine-induced cell killing, which is suggestive of an apoptosis-like death process.

[12] Khademvatan S, Gharavi MJ, and Saki J. Miltefosine induces metacaspase and PARP genes expression in *Leishmania infantum*. Braz J Infect Dis (2011) 15: 442–448.

[13] Moreira W, Leprohon P, and Ouellette M. Tolerance to drug-induced cell death favors the acquisition of multidrug resistance in *Leishmania*. Cell Death Dis 2011; 2: e201.

[14] Verma NK and Dey CS. Possible mechanism of miltefosine-mediated death of *Leishmania donovani.* Antimicro Agents Chemo (2004) 48 (8): 3010-3015.

Figure 13: Leishmaniacidal activity of miltefosine on *L. donovani* promastigotes.



(A) Cell viability of promastigotes treated with or without different concentrations of miltefosine was measured by MTT assay. Results are expressed as the means ± SEM (error bars) of three independent experiments.

(B) Cell number (live and moving) of promastigotes treated with or without miltefosine (25 μM) at different time points after miltefosine treatment. Results are expressed as the means ± SEM (error bars) of three independent experiments.

(C) Promastigotes at different time points after incubation with miltefosine (25 μM).

In another experiment, the effect of miltefosine exposure on cleavage of poly (ADP-ribose) polymerase (PARP) was evaluated. PARP is a DNA repair enzyme that undergoes cleavage during the process of induction of apoptosis. The presence of cleaved PARP is a feature of apoptosis. Cells incubated with or without the IC_{50} of miltefosine showed no cleavage of PARP under the experimental conditions tested.

In another experiment, the effect of incubation with miltefosine on nuclear condensation in promastigotes was tested; degradation of nuclear DNA into nucleosomal units is considered one of the hallmarks of apoptotic cell death. Oligonucleosomal-DNA fragmentation analysis of promastigotes incubated with 25 μM (10.2 μg/mL) miltefosine showed clear fragmentation of genomic DNA into oligonucleosomal fragments in the characteristic ladder form in agarose gel electrophoresis compared to promastigotes in drug free cultures.

Promastigotes incubated with 25 μM (10.2 μg/mL) miltefosine showed TdT-labeled nuclei (*in situ* TUNEL assay), which brightly fluoresced yellowish green, suggesting DNA fragmentation, compared to untreated promastigotes, which did not show any TUNEL-positive cells. Overall, the results suggest that miltefosine causes death of *L. donovani* promastigotes by inducing an apoptosis-like process.

Similar observations were made against the amastigote stage of *L. donovani*. Peritoneal macrophages isolated from Chinese hamsters were infected *in vitro* with amastigotes and

cultured in the presence (25 µM i.e., 10.2 µg/mL) or absence of miltefosine. Incubation with miltefosine killed intracellular *L. donovani* amastigotes as detected by the reduction in the number of intracellular amastigotes per macrophage by Giemsa staining. The amastigote-infected macrophages incubated with or without miltefosine (25 µM i.e., 10.2 µg/mL) were subjected to an *in situ* TUNEL assay. The nuclear DNA fragmentation of intracellular amastigotes, as determined by the green fluorescence, was clearly visible inside the infected macrophages incubated with miltefosine compared to the drug free cultures of amastigotes, which were not stained green. Macrophage nuclei were stained red, suggesting that no damage to the macrophage nuclei was caused by miltefosine at this concentration.

Overall, the results confirm that miltefosine induces apoptosis-like death process in *L. donovani*.

3.1.6. Immunomodulatory effects

The contribution of immunomodulatory activity of miltefosine to its anti-leishmanial activity is unclear. For example, miltefosine was shown to exhibit immunostimulatory properties that include

- induction of T-cell activation and cytokine release such as IL-2.
- enhancement of interferon-γ receptors and the Th1/Th2 balance in infected macrophages by promoting the interleukin-12-dependent Th1 response.

On the other hand, miltefosine was shown not to up-regulate major histocompatibility complex II or any co-stimulatory molecules that influence the maturation of dendritic cells, nor did it alter the release of the cytokines IL-10, IL-12, or TNF-α. Miltefosine inhibited both the release of mediators from mast cells as well as the related mast cell activation.

Comments:
Overall, the studies show that miltefosine has multiple sites of action (Figure 14) that include

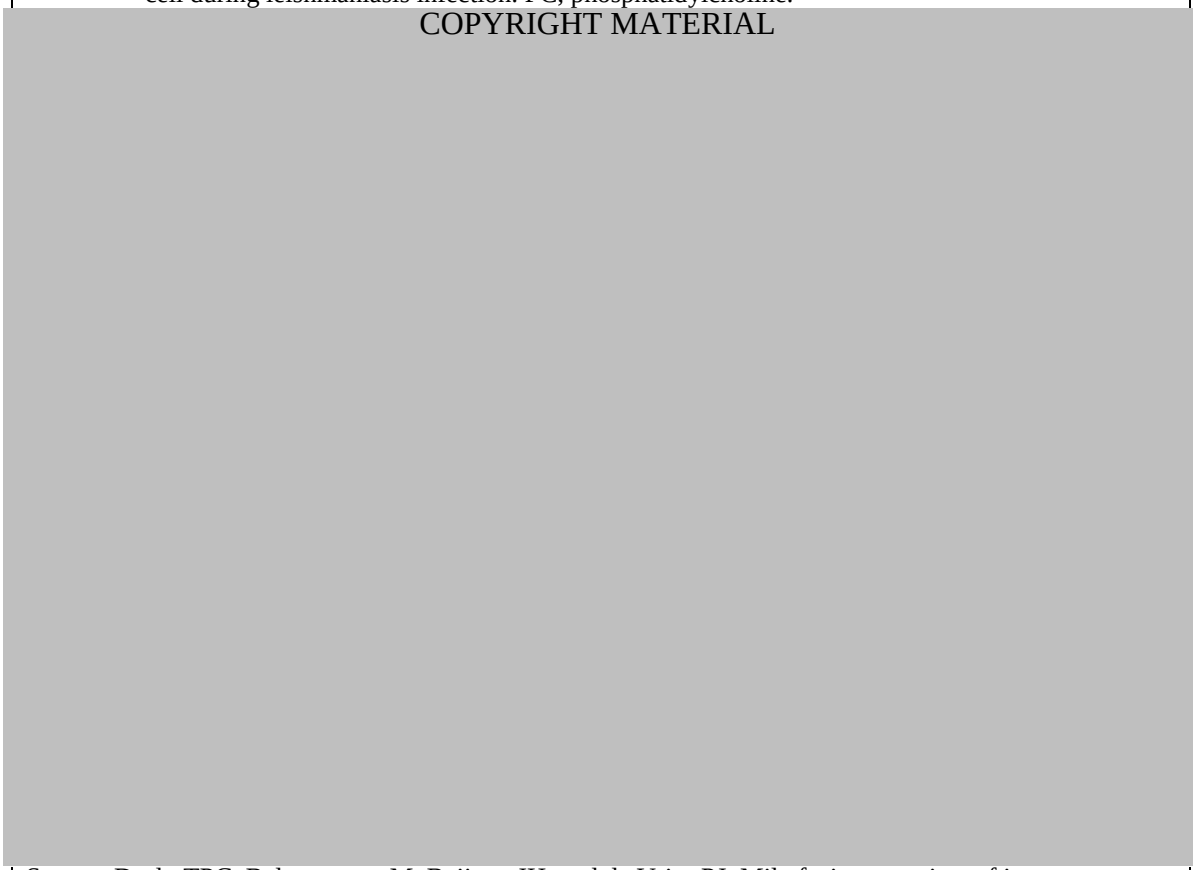
- *effect on interaction with lipids (phospholipids and sterols), including membrane lipids,*
- *mitochondrial effects especially inhibition of cytochrome c oxidase, and*
- *apoptosis-like cell death.*

- ***Effect on interaction with lipids (phospholipids and sterols), including membrane lipids***
  - *Miltefosine was shown to inhibit the alkyl-phospholipid biosynthesis by inhibiting acyl-CoA transferase activity and perturbing the later steps of alkyl-phospholipid biosynthesis of L. mexicana promastigotes. It is unclear if this pathway is the primary target. Most of the investigators stated that this pathway may not be the primary target as such changes were observed at high miltefosine concentration (50 µM i.e., 20.4µg/mL). The clinical pharmacology studies showed Cmax and AUC to be 37 to 72 µg/mL and 884 to 1344 µg/mL, respectively (see section 4, Overview of Clinical Pharmacology).*
  - *Miltefosine inhibited choline transport of L. major promastigotes.*
  - *Miltefosine was shown to interact with lipid monolayers in vitro; such an effect may depend on critical micellar concentration (CMC). High affinity of the drug for sterols was reported. In addition, miltefosine did not act as detergent disturbing membrane integrity.*
  - *Miltefosine was shown to effect fatty acid and sterol metabolism of promastigotes of a L. donovani strain.*

- ***Effect on mitochondria***
  - *Direct and dose-dependent inhibition of cytochrome c oxidase activity by miltefosine of transfected promastigotes of L. donovani was reported.*
  - *In L. panamensis promastigotes, miltefosine reduced the mitochondrial membrane potential.*
- ***Apoptosis-like cell death***

  Apoptosis-like effect was shown in L. donovani promastigotes and amastigotes. Inhibitory effect of miltefosine was associated with morphological changes (loss of cell volume), and DNA fragmentation with preservation of plasma membrane integrity. In one study, miltefosine, at the IC_{50} values, showed no cleavage of poly (ADP-ribose) polymerase under the experimental conditions tested. Apoptosis like cell death was also reported for promastigotes of L. infantum.

Figure 14: Anti-leishmanial mechanism of action of miltefosine. The various proposed mechanisms of action of miltefosine against the (intracellular) *Leishmania* parasite and the macrophage host cell during leishmaniasis infection. PC, phosphatidylcholine.



Source: Dorlo TPC, Balasegaram M, Beijnen JH, and de Vries PJ. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis J Antimicrob Chemother July 24, 2012 ejournal

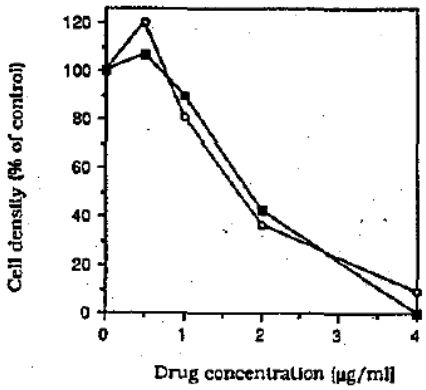
3.2. Activity *in vitro*

The activity of miltefosine was measured *in vitro* against the promastigotes and amastigote stages of different *Leishmania* species. A majority of the studies were based on testing of *L. mexicana* and *L. donovani* strains.

3.2.1. Activity against the promastigote forms

In a study by Lux *et al*. (1996),[3] the activity of miltefosine was measured *in vitro* against strain MNYC/BZ/62/M379 of ***L. mexicana*** promastigotes grown in a modified SDM growth media (SDM 93) at 26°C (strain MNYC/BZ/62/M379). Details of the method were not included in the publication. The results showed a stimulation of promastigote proliferation of up to 20% at low concentrations (~0.5 µg/mL) and inhibition of proliferation at higher concentrations (Figure 15); the IC_{50} values at 24 and 48 hours of incubation were 1.2 and 1.3 µg/mL, respectively.

Figure15: Effect of miltefosine on the proliferation of *L. mexicana* promastigotes.



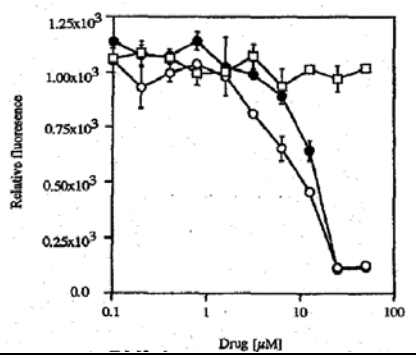
Promastigotes were seeded at 5 x 10^5 cells/mL and cell density determined after 24 hours (○) and 48 hours {•).

	LD_{50}*			
	24 hrs		48 hrs	
Compound	µg/ml	µM	µg/ml	µM
Pentostam	>100	—	>100	—
Pentamidine	1.4	—	0.4	—
Aminosidine	7.4	—	5.2	—
2-LPC	>16	>31	>16	>31
L-PAF	13	27	14	28
ET 18 OCH_3	1.6	3.0	1.5	2.8
ETPA	>58	>128	>58	>128
HePC	1.2	2.9	1.3	3.2
DH-10	1.3	na	1.2	na
DH-11	1.2	na	1.1	na
DH-12	0.9	na	0.8	na
DH-13	0.7	na	0.7	na
DH-14	>128	na	>128	na
DH-15	1.3	na	1.4	na
DH-16	1.5	na	1.5	na
DH-17	1.3	na	1.5	na
DH-18	2.0	na	1.6	na

HePC=miltefosine

In another study by Lux *et al.* (2000)[4] the *in vitro* sensitivity was measured against the promastigotes of 2 strains (MHOWBZ/84/BEL46 and MNYC/BZ/62/M379) of ***L. mexicana***. After 72 hours of incubation of promastigotes (10^5/mL) in SDM with the drug at 28°C, Alamar Blue™ was added into each well and the plates incubated for 3 hours before viability was determined using a cytofluorimeter at 530 nm/590 nm and the IC_{50} values determined. The results showed miltefosine to be effective in inhibiting the growth of promastigotes in culture with IC_{50} value of 14 µM i.e., 5.7 µg/mL (Figure 16).

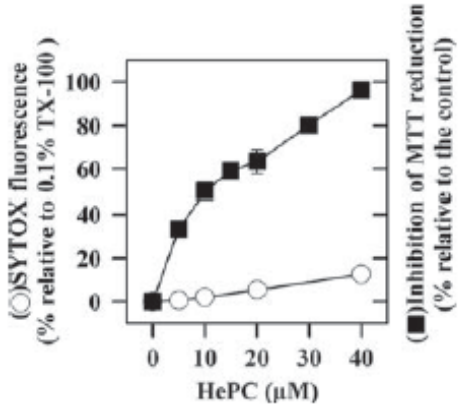
Figure 16: Viability of *L. mexicana* promastigotes after incubation with miltefosine as detected by Alamar Blue.



Cell viability after 72 hours in the absence (open squares) or presence of different concentrations (µM) of miltefosine (open circles) and edelfosine (closed circles). Data represents two independent experiments each carried out in duplicate.

A study by Luque-Ortega *et al*. (2007[10]), reported the effect of different concentrations of miltefosine on viability and permeability of promastigotes of ***L. donovani*** strain MHOM/SD/00/1S-2D by incubating cultures for 14 hours at 25°C. Viability was measured by reduction of MTT to formazan. Permeability was measured by incorporation of a cationic vital dye SYTOX green into the cytoplasm and Polarstar Galaxy microplate reader at 485- and 520-nm wavelengths, respectively. A dose-dependent inhibition of cell proliferation was observed, with an IC_{50} of 10.2 ± 0.5 µM (i.e., 4.2 ± 0.2 µg/mL) and full inhibition at 40 µM i.e., 16.3 µg/mL (Figure 17). However, the level of SYTOX green fluorescence attained was only 10% whereas TX-100 used as a positive control fully permeabilized the membrane.

Figure 17: Effect of miltefosine on plasma membrane permeabilization and viability of *L. donovani* promastigotes



Plasma membrane permeabilization and viability of *L. donovani* promastigotes after incubation for 14 hours with miltefosine. The entry of SYTOX green after incubation with miltefosine (HePC) was monitored by determination of the increase in fluorescence (excitation λ, 485 nm; emission λ, 520 nm). The results were normalized as the percentage of fluorescence relative to that obtained by maximal parasite permeabilization with 0.1% TX-100. The inhibition of parasite viability was assessed by determination of the inhibition of MTT reduction.

In a study by Kuhlencord *et al*. (1992),[15] the activity of miltefosine was reported against the promastigotes of ***L. donovani*** strains MHOM/IN/54/LRC-L.51 (LRC-L.51) and MHOM/IN/80/DD8 (DD8) and ***L. infantum*** strain MHOM/ES/86/STI-172 (STI-172). All strains were isolated from patients with visceral leishmaniasis and stabilates of these strains stored in liquid nitrogen. Before they were used for experiments, the strains were passaged through BALB/c mice by intravenous injection. Promastigote cultures were maintained at 26°C in RPMI 1640 medium with and without miltefosine for 96 hours and number of promastigotes determined in a Neubauer counting chamber. The IC_{50} was calculated graphically. Experiments were repeated three times, and each set of three experiments was done in duplicate. The miltefosine IC_{50} values were between 0.89 and 2.25 µg/mL and IC_{90}s between 2.60 and 4.90 µg/mL (Table 3).

Table 3: *In vitro* sensitivity of *L. donovani* and *L. infantum* strains to miltefosine

Strain	**IC_{50} (µg/mL)** (mean ± SD)	**IC_{90} (µg/mL)** (mean ± SD)
L. donovani (LRC-L.51)	2.07 ± 3.13	3.13 ± 0.29
L. donovani (DD8)	0.89 ± 0.08	2.60 ± 0.07
L. infantum (STI-172)	2.25 ± 0.21	4.90 ± 0.40

In a study by Unger *et al*., 1998[16], the activity of miltefosine against the promastigotes forms of ***L. donovani*** strain MHOM/IN/54LRC-L.51 was reported. Promastigotes were cultured in 24-well-plates. After 96 hours of incubation in control or drug-containing media the number of promastigotes were determined in a Neubauer counting chamber. The IC_{50} value, calculated graphically, was 2.07 ± 0.23 µg/mL.

In another study (Escobar *et al*., 2002[17]), the activity of miltefosine was reported against the promastigote stages of six species of *Leishmania*: ***L. donovani*** (MHOM/ET/67/L82), ***L. major*** (MHOM/SA/85/JISH118), ***L. tropica*** (MHOM/AF/82/KOO1), ***L. aethiopica*** (MHOM/ET/84/KH), ***L. mexicana*** (MHOM/BZ/82/BEL21) and ***L. panamensis*** (MHOM/PA/67/BOYNTON). The promastigotes (10^6/mL) were cultured in Schneider's medium at 26°C for all the *Leishmania* species except *L. donovani* that were cultured in M199 medium at 26°C. Different concentrations of miltefosine were added and cultures incubated for 72 hours at 26°C. Inhibition of promastigote growth was determined microscopically by counting parasite numbers in a Neubauer haemocytometer. Inhibition of growth was determined by comparison to drug free controls.

The miltefosine IC_{50} values against the promastigote stage ranged from 0.36–13.1 µM i.e., 0.15 – 5.4 µg/mL. The miltefosine IC_{50} value was lowest against *L. donovani* compared to other *Leishmania* species (Table 4). The order of sensitivity to miltefosine of promastigotes was *L. donovani* > *L. aethiopica* > *L. tropica* > *L. panamensis* > *L. mexicana* > *L. major*, with *L. major* being significantly less sensitive (P<0.05) than *Leishmania* species other than *L. mexicana*.

[15] Kuhlencord A, Maniera T, Eibl H, and Unger C. Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. Antimicrob Agents Chemother (1992) 36: 1630-1634.

[16] Unger C, Maniera T, Kaufmann P and Eibl H. *In vivo* antileishmanial activity of hexadecylphosphocholine and alkylphosphocholines. Drugs of Today (1998) 34 (Suppl F): 133-140.

[17] Escobar P, Matu S, Marques C, and Croft SL. Sensitivities of *Leishmania* species to hexadecylphosphocholine (miltefosine), ET-18-OCH3 (edelfosine) and amphotericin B. Acta Tropica (2002) 81: 151-157.

Table 4: Activity against promastigotes and amastigotes of *Leishmania* species

Promastigotes

Leishmania spp	*n*	HPC	ET-18-OCH_3	Amphotericin B
L. donovani	1	0.45 (0.18–0.71)	0.15 (0.11–0.19)	0.012 (0.010–0.150)
	2	0.36 (0.27–0.44)	1.32 (0.36–2.28)	0.003 (0.0030–0.0033)
L. aethiopica	1	2.76 (2.13–3.39)	1.28 (0.99–1.58)	0.110 (0.110–0.111)
	2	1.16 (0.87–1.45)	0.62 (0.54–0.71)	0.239 (0.090–0.386)
L. tropica	1	1.74 (1.63–1.86)	0.59 (0.36–0.84)	0.053 (0.052–0.054)
	2	0.55 (0.17–0.93)	0.52 (0.09–1.01)	0.084 (0.081–0.087)
L. mexicana	1	2.37 (1.33–3.41)	3.11 (1.25–4.97)	0.27 (0.25–0.29)
	2	12.71 (4.30–21.10)	2.91 (0.46–5.76)	0.22 (0.09–0.35)
L. panamensis	1	1.30 (1.04–1.56)	2.23 (1.26–3.19)	0.037 (0.0370–0.0372)
	2	3.72 (1.36–6.10)	1.94 (1.10–2.79)	0.052 (0.018–0.087)
L. major	1	13.10 (12.03–13.51)	1.42 (0.98–1.86)	0.043 (0.0435–0.0437)
	2	4.80 (0.93–8.61)	0.50 (0.10–0.90)	0.040 (0.033–0.047)

ED_{50} values in μM with P_{95} confidence limits; *n*, number of experiment.

Amastigotes

Leishmania spp	*n*	% Mϕs	HPC	ET-18-OCH_3	Amphotericin B
L. donovani	1	87.0	4.56 (3.72–5.70)	2.49 (1.89–3.09)	0.036 (0.026–0.046)
	2	80.0	3.32 (2.82–3.82)	1.17 (0.87–1.47)	0.05 (0.041–0.059)
L. aethiopica	1	84.5	4.92 (4.16–5.97)	2.92 (2.71–3.73)	0.07 (0.058–0.076)
	2	74.3	2.63 (2.36–2.94)	1.15 (1.07–1.22)	0.039 (0.031–0.047)
L. tropica	1	85.1	5.82 (4.83–6.84)	2.00 (1.59–2.38)	0.05 (0.009–0.013)
	2	46.0	10.23 (7.24–12.95)	5.71 (3.79–7.80)	0.09 (0.082–0.098)
L. mexicana	1	95.6	6.83 (5.77–7.80)	3.94 (2.68–5.21)	0.14 (0.131–0.153)
	2	72.1	10.12 (4.35–15.87)	5.83 (4.28–7.37)	0.12 (0.115–0.125)
L. panamensis	1	95.7	10.63 (9.78–11.45)	4.9 (4.40–5.42)	0.08 (0.081–0.082)
	2	73.0	10.63 (9.38–11.58)	6.97 5.95–8.08	0.13 (0.05–0.21)
L. major	1	93.8	37.17 (35.40–38.82)	7.48 (5.22–9.87)	0.05 (0.049–0.053)
	2	73.1	31.56 (25.52–36.15)	8.80 (7.84–9.45)	0.05 (0.041–0.059)

ED_{50} values in μM with P_{95} confidence limits; *n*, number of experiment; %MΦs = %macrophages infected in untreated control cultures at 72 h.

HPC=miltefosine

Amphotericin B was more active than miltefosine against all *Leishmania* species.

In another study (Verma and Dey, 2004[14]), the activity of miltefosine was reported against the promastigotes of ***L. donovani*** (strain MHOM/80/IN/Dd8) by the MTT assay as summarized above (see mechanism of action section 3.1). The results showed a slow killing effect up to 20 µM (8.2 µg/mL) concentration and a rapid and dose-dependent cell death occurred at higher miltefosine concentrations (30 and 50 µM i.e., 12.2 - 20.4 µg/mL), reaching approximately 100% at around 40 µM (16.3 µg/mL). The IC_{50} was 25 µM i.e., 10.2 µg/mL (Figure 13).

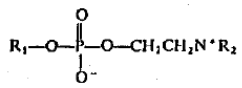
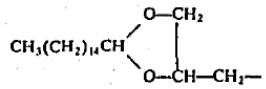
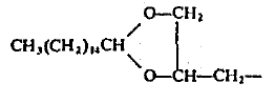
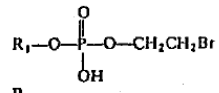
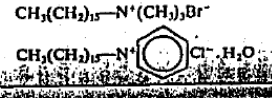
In a study by Croft *et al.* (1987),[18] the activity of miltefosine was measured against the promastigotes stage by culturing the amastigotes in Schneider's medium plus 20% heat-inactivated FCS at 26^{o}C. The parasites were diluted to 2 x 10^{6}/mL in the same medium in a microtiter plate. Experimental drugs were added to give a final concentration of 50, 10 and 2 µg/mL and cell motility and viability monitored through an inverted microscope for 48 hours. The authors state that activity against promastigotes was similar to that reported against amastigotes (see below) but data were not shown.

3.2.2. Activity against the amastigote forms

In a study by Croft *et al.* (1987),[18] the activity of miltefosine was reported against amastigotes isolated from the spleen of mice infected with ***L. donovani*** (strain MHOM/ET/67/L82; LV9). Peritoneal macrophages from CD-1 mice were infected with freshly isolated amastigotes and incubated with different concentrations of several alkyl phosphocholines 24 hours later and cultures incubated for 7 days; the medium containing drug was changed 2 times during the 7 day period. The proportion of infected macrophages in Giemsa-stained preparations was determined after the 7-day exposure to drugs and IC_{50} values calculated by linear regression analysis. The miltefosine IC_{50} value was 11.4 µM i.e., 4.6 µg/mL (Table 5). The alkylphosphocholines showed selective toxicity to the amastigotes compared with their mammalian host cells (the concentration tolerated by macrophages was approximately 5-fold higher than the IC_{50} value.

[18] Croft SL, NEAL RA, Pendergast W, and Chan JH. The activity of alkyl phosphorylcholines and related derivatives against L*eishmania donovani.* Biochem Pharmacol (1987) 36: 2633-2636.

Table 5: Activity of compounds against *Leishmania donovani* amastigotes *in vitro*

Compound number	Compound structure		Max. conc^n tolerated by macrophages (mg/l)	ED_{50} mg/l (P_{95} fiducial limits)	[μM]
Group 1 alkyl phosphorylcholines					
	R_1	R_2			
I	$CH_3(CH_2)_{15}$—	$(CH_3)_3$	27	5.0 (5.4–4.7)	[11.39]
II	$CH_3(CH_2)_{17}$—	$(CH_3)_3$	9	1.5 (1.4–1.6)	[3.08]
III	$CH_3(CH_2)_{13}CH(CH_3)$—	$(CH_3)_3$	9	1.2 (1.4–0.9)	[2.95]
IV	$CH_3(CH_2)_{15}CH(CH_3)$—	$(CH_3)_3$	9	5.3 (6.0–4.7)	[11.55]
V	$CH_3(CH_2)_{17}CH(C_2H_5)CH_2$—	$(CH_3)_3$	9	0.5 (0.6–0.4)	[0.97]
VI	$CH_3(CH_2)_{17}CH(C_3H_7)CH_2$—	$(CH_3)_3$	9	1.4 (1.6–1.2)	[2.63]
VII		$(CH_3)_3$	9	4.1 (4.8–3.5)	[8.25]
VIII		$(H_3)_3$	9	2.3 (2.6–2.1)	[5.25]
Group 2 alkyl phosphorylbromoethanol derivatives					
	R_1				
IX	$CH_3(CH_2)_{13}CH(CH_3)$—		27	no activity at 27 mg/l	
X	$CH_3(CH_2)_{16}CH_2$—		27	no activity at 27 mg/l	
Group 3 detergents					
XI	$CH_3(CH_2)_{15}$—$N^+(CH_3)_3Br^-$		0·3	0.16 (0.3–0.1)	[0.44]
XII			0.1	0.02 (0.02–0.015)	[0.056]

In another study by Croft *et al*. (1996[19]), the activity of miltefosine was measured against *L. donovani* strains MHOM/ET/67/L82 and MHOM/IN/82/Nandi II. These strains were routinely maintained in golden hamsters by passage every 6 to 8 weeks. Amastigotes were isolated from the spleen of hamsters and used to infect the macrophage cultures from the spleen of CD-1 mice and maintained at 37°C as summarized above. The IC_{50} values were determined by linear regression analysis. The results showed IC_{50} to be 0.2 µM and 3.9 µM (i.e., 0.08 and 1.6 µg/mL) against the Nandi II (Indian) and L82 (Ethiopian) strains, respectively (Table 6).

[19] Croft SL, Snowdon D, and Yardley V. The activities of four anticancer alkyllysophospholipids against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei*. J Antimicro Chemotherapy (1996) 38: 1041-1047.

Table 6: The activities of miltefosine and other analogues against *L. donovani*, *T. cruzi* and *T. brucei in vitro*

Parasite and ALPs	90[b]	% inhibition (concn in μM)[a] 30	10	3	1	0.3	ED_{50} values (μM)
L. donovani (LV9)							
ilmofosine	NT	99.7	92.3	37.1	32.5	11.5	2.6
miltefosine (HDPC)	NT	97.9	86.2	27.2	6.3	4.6	3.9
edelfosine (ET-18-OCH_3)	NT	98.5	72.0	14.2	14.5	6.9	5.0
SRI 62-834	NT	T[c]	68.0	41.9	16.6	0.7	4.4
L. donovani (Nandi II)							
ilmofosine	NT	100.0	93.7	87.9	52.8	NT	0.6
miltefosine (HDPC)	NT	100.0	100.0	89.5	70.5	NT	0.2
edelfosine (ET-18-OCH_3)	NT	100.0	99.2	92.3	50.9	NT	0.7
SRI 62-834	NT	T[c]	99.7	59.6	20.6	NT	2.3
T. cruzi (Y)							
ilmofosine	NT	99.7	97.3	94.5	85.3	34.2	0.2
miltefosine (HDPC)	NT	99.7	94.9	76.0	56.8	42.4	0.5
edelfosine (ET-18-OCH_3)	NT	99.2	85.7	84.1	49.6	8.1	1.4
SRI 62-834	NT	T[c]	98.6	77.4	60.6	10.1	1.5
T. brucei brucei (S427)							
ilmofosine	100	100	64.8	7.7	0	NT	7.0
miltefosine (HDPC)	100	13.2	7.5	0	0	NT	35.5
edelfosine (ET-18-OCH_3)	100	5.8	0	0	0	NT	44.0
SRI 62-834	100	71.0	15.7	14.9	0	NT	18.2
T. b. rhodesiense (STIB 900)							
ilmofosine	100	100	0	0	0	NT	18.1
miltefosine (HDPC)	100	0	0	0	0	NT	47.0
edelfosine (ET-18-OCH_3)	100	33.7	0	0	0	NT	40.7
SRI 62-834	100	0	0	0	0	NT	50.8

[a]Data from one test shown.
[b]Not tested at 90 μM against *L. donovani* or *T. cruzi* as toxic to macrophages.
[c]Toxic to macrophages.
NT. Not tested.

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In another study (Escobar *et al*., 2001[20]), the activity of miltefosine was reported against *L. donovani* (strain no specified) amastigotes using peritoneal macrophages from BALB/c and *scid* mice. Adherent peritoneal macrophages were infected with *L. donovani* amastigotes at a ratio of 10 parasites:1 macrophage. After 12 hours, nonphagocytosed parasites were removed by washing with serum-free DMEM. Infected cultures were incubated for 72 hours with different concentrations of miltefosine. Drug activity was determined microscopically by counting the percentage of infected cells in methanol-fixed and Giemsa stained preparations. No difference was observed in the activity of miltefosine in peritoneal macrophages from scid or BALB/c mice (Table 7). In contrast, significant differences (P >0.05) were observed in the anti-leishmanial activity of Pentostam, with IC_{50}s for scid mouse derived infected peritoneal macrophages being approximately three-fold higher than those for BALB/c mouse derived peritoneal macrophages. AmBisome was more active in BALB/c mouse peritoneal macrophages than in scid mouse peritoneal macrophages.

[20] Escobar P, Yardley V, and Croft S. Activities of hexadecylphosphocholine (miltefosine), AmBisome, and sodium stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficient scid mice. (2001) Antimicrob Agents Chemother 45: 1872-1875.

Table 7: Activities of HPC, AmBisome, and Pentostam against *L. donovani* in resident peritoneal macrophages from BALB/c and scid mice

Compound	BALB/c mice		*scid* mice	
	ED_{50}[a]	ED_{90}[a]	ED_{50}	ED_{90}
HPC	7.48 (7.29)[b]	10.85 (10.31)	7.47 (11.12)[c]	11.42 (20.30)
Pentostam	7.58 (10.5)	13.15 (>25)	20.69 (>25)[d]	>25 (>25)
AmBisome	0.05	0.2	0.11[d]	>0.25
Fungizone	0.03	0.061	0.06[d]	>0.12

[a] The ED_{50}s and ED_{90}s are in micromolar for HPC, AmBisome, and Fungizone and are in micrograms of Sb^V per milliliter for Pentostam.
[b] The values parentheses are the results of a second experiment. *P* values were calculated for the drug activity after 72 h, in infected PMϕ from BALB/c and *scid* mice.
[c] $P = 0.25$.
[d] $P > 0.05$.

HPC=miltefosine

In another study (Escobar *et al*., 2002[17]), the activity of miltefosine was measured against the amastigote stages of six species of *Leishmania*: *L. donovani* (MHOM/ET/67/L82), *L. major* (MHOM/SA/85/JISH118), *L. tropica* (MHOM/AF/82/KOO1), *L. aethiopica* (MHOM/ET/84/KH), *L. mexicana* (MHOM/BZ/82/BEL21) and *L. panamensis* (MHOM/PA/67/BOYNTON). Adherent peritoneal macrophages from the peritoneal cavity of induced (with soluble starch) CD-1 mice were infected with stationary phase promastigotes at a ratio of 10:1. Cultures were incubated at 34°C for all *Leishmania* species except *L. donovani* that were incubated at 37°C. After 16 hours, non-phagocytized parasites were removed by washing and infected cultures incubated for 72 hours with or without miltefosine and other drugs at different concentrations. Drug activity was determined from the percentage of infected cells in treated and untreated cultures in methanol fixed and Giemsa stained preparations and ED_{50} and ED_{90} values calculated by sigmoidal regression analysis.

The miltefosine IC_{50} values against the amastigote stage ranged from 2.6–37.2 µM (1.1 – 15.2 µg/mL) and were higher (2 to 10-fold) than miltefosine IC_{50} values against the promastigote stage (Table 4). The order of sensitivity to miltefosine of amastigotes was *L. donovani* > *L. aethiopica* > *L. tropica* > *L. mexicana* > *L. panamensis* > *L. major* with *L. major* being significantly less sensitive ($P<0.05$) than other species tested. Amphotericin B appears to be more active than miltefosine against all *Leishmania* species.

Morais-Teixeira *et al*. (2011),[21] reported *in vitro* susceptibility testing of miltefosine against the amastigote forms of four *Leishmania* species found in Brazil (*L. chagasi* strain MHOM/BR/70/BH46, *L. amazonensis* strain IFLA/BR/1967/PH-8, *L. braziliensis* strain WHO-MHOM/BR/75/M2903, *L. guyanensis* strain MHOM/BR/1997/321-P). *L. donovani* strain MHOM/ET/1967/HU3 was included as a comparator. These strains were initially passaged in animals and amastigotes of *L. chagasi* and *L. donovani* harvested from spleens of infected golden hamsters; *L. amazonensis*, *L. braziliensis* or *L. guyanensis* amastigotes were harvested from the skin lesions. Activated peritoneal macrophages from BALB/c mice were infected with 4 x 10^6 amastigotes in the ratio of 1:10. Different concentrations of miltefosine were added after 4 hours of culture at 37°C and washing to remove free-floating parasites; the cultures were incubated for 72 hours at 37°C. The percentage of infected cells was counted after Giemsa staining. The results

[21] Morais-Teixeira E de, Damasceno QS, Galuppo MK,, Romanha AJ, and Rabello A. The *in vitro* leishmanicidal activity of hexadecylphosphocholine (miltefosine) against four medically relevant *Leishmania* species of Brazil. *Mem Inst Oswaldo Cruz*, Rio de Janeiro (2011) 106 (4): 475-478.

were presented as the ratio of infection (number of amastigotes) between the treated and non-treated macrophage cultures and expressed as IC_{50} and IC_{90} values.

The Alamar Blue™ micromethod was used to estimate the 50% cytotoxicity concentration (CC_{50}) of miltefosine in macrophages after 72 hours of incubation. The selectivity index (SI) was determined (CC_{50}/ IC_{50}).

The miltefosine IC_{50} against *L. donovani* was the lowest and was roughly 20-fold lower than the IC_{50} determined for *L. chagasi* (p = 0.03). The miltefosine IC_{50} for *L. amazonensis*, *L. braziliensis*, and *L. guyanensis* were higher than *L. donovani* and similar to *L. chagasi* (Table 8).

The CC_{50} of miltefosine against macrophages was 37.8 µg/mL (92.7 µM). A ratio (SI) of cytotoxicity to biological activity (CC_{50}/IC_{50}) against different *Leishmania* species ≥ 17 (Table 8).

Table 8: Inhibitory concentrations (IC_{50} and IC_{90}) and selectivity index (SI) of miltefosine against intracellular amastigotes of five *Leishmania* species

	IC_{50}		IC_{90}				
Leishmania spp	µM	µg/mL (CI[a])	µM	µg/mL (CI[a])	Infection level[b] [% (± SD)]	SI[c]	R^2
L. (L.) amazonensis	3.21	1.31 (0.75 - 1.89)	7.88	3.21 (2.19 - 4.24)	84.3 (4.5)	28.85	0.798
L. (V.) braziliensis	5.40	2.20 (1.54 - 2.90)	13.91	5.67 (4.67 - 6.67)	81 (0.5)	17.18	0.765
L. (V.) guyanensis	4.02	1.64	14.84	6.05	84.2 (2.6)	26.05	-[d]
L. (L.) chagasi	4.46	1.82 (0.65 - 3.00)	12.39	5.05 (3.27 - 6.82)	83.1 (3.0)	20.77	0.713
L. (L.) donovani	0.22	0.09 (0.03 - 0.014)	0.52	0.21 (0.14 - 0.27)	80 (0.0)	420.0	0.700

a: confidence interval (CI) 95%; *b*: infection level indicates the percentage of macrophages infected in untreated control culture; *c*: selectivity index (SI) = CC_{50}/IC_{50}; *d*: IC_{50} and IC_{90} determined by linear interpolation; R^2: coefficient of linear regression; SD: standard deviation.

3.2.3. Activity against clinical isolates

The *in vitro* activity of miltefosine was assessed against the **amastigotes** of ***L. donovani*** clinical isolates (n=28) obtained from patients with visceral leishmaniasis from 2 regions known to be of low (eastern Uttar Pradesh region with few cases per 10,000 populations; n=12) and high (Bihar, region with ≥ 30 cases per 10,000 populations; n=16) endemicity in India (Prajapati *et al.*, 2012).[22] Extensive use of miltefosine has been reported in the high endemic area. Diagnosis of visceral leishmaniasis was parasitologically confirmed by demonstration of amastigotes in splenic smears. Splenic aspirates obtained prior to initiation of therapy were cultured at 26°C and used for *in vitro* testing after 2 to 3 passages. *Leishmania* species identified was *L. donovani* by restriction fragment length polymorphism (RFLP) of the HSP70 gene. The promastigotes (5 x 10^5) were added to adherent macrophages (J774A.1 macrophage cell line) in the ratio of 10:1 and incubated for 24 hours at 37°C. Non-internalized promastigotes were removed, and infected cells incubated with different concentrations of miltefosine (1 to 25 µg/mL) and other comparator drugs. Cultures were incubated at 37°C for 72 hours and intracellular amastigotes (infected

[22] Prajapati VK, Mehrotra S, Gautam S, Tai M, and Sundar S. *In vitro* antileishmanial drug susceptibility of clinical isolates from patients with Indian visceral leishmaniasis—status of newly introduced drugs. Am J Trop Med Hyg (2012) 87 (4): 655-657.

macrophages) were counted after Giemsa staining. The percentage inhibition relative to untreated macrophages was calculated on the basis of the comparison of total amastigotes per 100 macrophages.

The level of infection obtained at 24 hours post-infection was 60–80% in plated macrophages. The infection level was approximately one to two amastigotes per macrophage. The results showed that miltefosine IC_{50} values were similar against isolates from low and high endemic regions (Table 9). However, the IC_{90} values were higher against isolates from subjects from high endemic region compared to low endemic area. There was no significant difference between the drug sensitivities of parasites to amphotericin B isolated from different endemic regions.

Table 9: ED_{50} and ED_{90} values for *in vitro* susceptibility of clinical isolates to anti-leishmanial drugs

Geographical status and drug concentration	Endemic region	Non-endemic region	*P* value
Miltefosine			
ED_{50} (μg/mL) amastigotes			
Range	5.3–10.9	3.5–10.1	0.08
Mean ± SD	7.1 ± 1.6	5.7 ± 2.2	
ED_{90} (μg/mL)			
Amastigotes	14.1–23.8	9.8–19.1	0.005
Mean ± SD	17.5 ± 2.4	13.7 ± 3.6	
Paromomycin			
ED_{50} (μg/mL) amastigotes			
Range	90.8–200.9	99.6–174.86	0.15
Mean ± SD	139.8 ± 33.6	122.1 ± 23.5	
ED_{90} (μg/mL)			
Amastigotes	163.8–380.3	189.8–265.7	0.02
Mean ± SD	279.6 ± 54.1	232.9 ± 24.8	
Amphotericin B			
ED_{50} (μg/mL) amastigotes			
Range	0.007–0.014	0.008–0.016	0.92
Mean ± SD	0.011 ± 0.002	0.011 ± 0.003	
ED_{90} (μg/mL)			
Amastigotes	0.023–0.031	0.019–0.035	0.05
Mean ± SD	0.027 ± 0.003	0.024 ± 0.005	
SSG			
ED_{50} (μg/mL) amastigotes			
Range	14.2–34.4	12.6–21.1	0.0007
Mean ± SD	24.1 ± 5.4	17.2 ± 2.9	
ED_{90} (μg/mL)			
Amastigotes	32.9–74.1	33.2–50.8	0.0001
Mean ± SD	56.6 ± 9.6	42.0 ± 5.1	

Nonendemic ~ low endemicity

In another study (Kulshrestha *et al*., 2013[23]), the activity of miltefosine against **the promastigote and amastigote stages of *L. donovani*** isolates was reported. Testing was performed in 2 laboratories. Briefly, splenic aspirates were collected from Indian patients with visceral leishmaniasis (n=17) and post-kala-azar dermal leishmaniasis (n=7) and cultured. Also, three miltefosine-induced parasite strains were included to evaluate the correlation with high miltefosine tolerance. For amastigote assay, J774A.1 cells were used for testing in one laboratory (Lab 1) and mouse peritoneal macrophages in another laboratory (Lab 2). Cultures in the presence or absence of the drug were incubated at 37°C for 48 hours and the number of infected macrophages counted after Giemsa staining.

[23] Kulshrestha A, Bhandari V, Mukhopadhyay R, Ramesh V, Sundar S, Maes L, Dujardin JC, Roy S, and Salotra P. Validation of a simple resazurin-based promastigote assay for the routine monitoring of miltefosine susceptibility in clinical isolates of *Leishmania donovani*. Parasitol Res (2013) 112:825–828.

For the promastigote assay, cultures, with and without drug, were incubated for 72 hours at 25°C and resazurin added. In one laboratory (Lab 1), cultures were incubated for additional 24 hours and cell viability measured by fluorometer at 550/590 nm. In the other laboratory (Lab 2), cells were incubated with Alamar Blue for 4 hours at 37°C and optical density measured at 570 nm. The results showed variability in IC_{50} values against the amastigote and promastigote stages of isolates from 24 subjects (Figure 18). However, there was a good correlation of the activity of miltefosine against the amastigote and promastigote stages in both laboratories (Figure 18). In Lab 1, the miltefosine IC_{50}s against the amastigotes in J774.A.1 macrophage cell line ranged from 0.3 to 24.9 µM (0.1 to 10.1 µg/mL). Range of miltefosine IC_{50} against the promastigote stage were 1.5 to 12.6 µM (0.6 to 5.1 µg/mL).

In Lab 2 for the same set of clinical isolates (n = 17), against the amastigote stage the miltefosine IC_{50} values in mouse peritoneal exudate cells, ranged from 0.03 to 6.1 µM (0.01 to 2.5 µg/mL). The miltefosine IC_{50}s against the forms promastigote ranged from 2.8 to 18.2 µM (1.1 to 7.4 µg/mL).

The differences in the IC_{50} values observed in the two labs could be the result of differences in the method (fluorometric in Lab 1 and colorimetric in Lab 2 for the promastigote assay and different macrophages for the amastigote assay).

The 3 miltefosine-induced *L. donovani* promastigotes (adapted to miltefosine 12, 37 and 49 µM i.e., 4.9, 15.1, and 20 µg/mL) showed evidence of higher IC_{50} values. It is unclear if the IC_{50} values represent testing in one laboratory or both laboratories.

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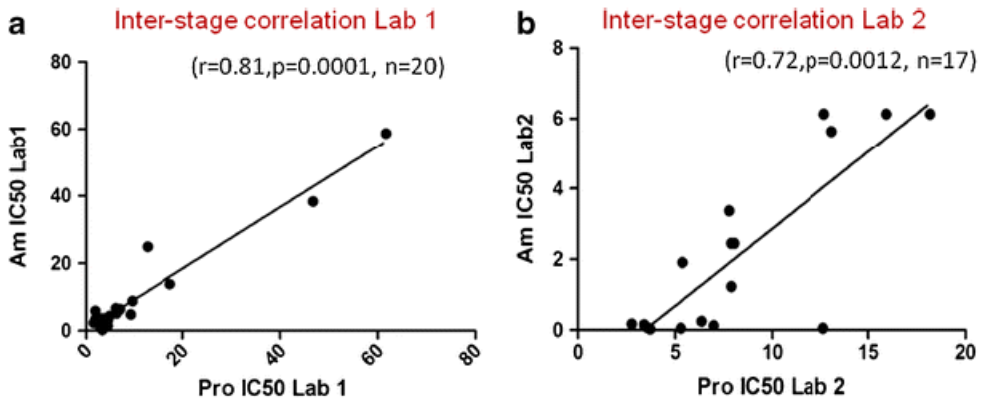
Figure 18: Inter-stage susceptibility of miltefosine-resistant parasites and PKDL isolates to miltefosine



S. no.	Parasite ID	IC_{50} (µM ± SD)	
		Promastigote	Amastigote
1	BHU573Mil-R(12 µM)	17.40 ± 3.70	13.80 ± 1.80
2	BHU573Mil-R(37 µM)	46.84 ± 1.00	38.03 ± 3.46
3	BHU573Mil-R(49 µM)	61.70 ± 2.50	58.80 ± 2.20
4	NIP/P44	3.12 ± 0.06	7.37 ± 0.07
5	NIP/P48	2.98 ± 0.28	8.88 ± 0.18
6	NIP/P49	4.06 ± 0.25	9.23 ± 0.22
7	NIP/P93	4.34 ± 0.91	9.69 ± 0.52
8	NIP/P232	4.28 ± 0.38	7.99 ± 0.15
9	NIP/P195R	8.50 ± 2.07	13.26 ± 0.89
10	NIP/P214R	6.76 ± 1.77	16.70 ± 0.65

The data represent IC_{50} mean ± SD of two independent experiments performed at least in duplicate.

S. nos. 1–3 represent visceral leishmaniasis parasite (BHU 573 clone-3) made resistant to different miltefosine concentrations (12, 37 and 49 µM miltefosine).

S. nos. 4–10 represent post-kala-azar dermal leishmaniasis (PKDL) isolates

Comments:

- *Other than L. donovani, the number of strains of different Leishmania species tested for in vitro sensitivity to miltefosine varied from 1 to 2 (Table 10 and 1). The highest IC_{50} value reported was 10.2 µg/mL against the promastigote forms of L. donovani strain DD8 and 15.2 µg/mL against the amastigote forms of L. major. Testing of clinical isolates was limited to L. donovani species and the IC_{50} values ranged from 0.6 to 7.4 µg/mL against the promastigote forms and 0.01 to 10.9 µg/mL against the amastigote forms; the IC_{90} values varied from 9.8 – 23.8 µg/mL against the clinical isolates of L. donovani.*

- *In one study, one strain each of 6 different Leishmania species was tested. The results suggested L. donovani to be the most sensitive species and L. major the least sensitive.*

However, such differences in the IC_{50} values were not apparent when compared across different studies; this could be due to

- *limited number of strains of different Leishmania species tested, or*
- *inter-laboratory variations due to differences in methods or operators and lack of standardization of the assays.*

- *In one study, the miltefosine IC_{50} values were shown to be similar against isolates from low and high endemic regions in India (Table 9). However, the IC_{90} values were higher against isolates from high endemic region compared to low endemic area suggesting miltefosine IC_{90} values may be more clinically relevant than the IC_{50} values. Activity (based on IC_{50} and IC_{90} values) of amphotericin B against isolates from different endemic regions was similar.*
- *In one study, the IC_{50} values showed a trend towards an increase against the amastigote forms compared to promastigotes.*
- *In one study, stimulation of promastigote proliferation of up to 20% at low concentrations (~0.5 µg/mL) and inhibition of proliferation at higher concentrations (≥1 µg/mL) was reported for one strain of L. donovani.*
- *The in vitro anti-leishmanial activity of miltefosine against the amastigote forms was similar in peritoneal macrophages derived from either scid or BALB/c mice, whereas Pentostam and AmBisome were significantly more active in cells from BALB/c mice than scid mice. However, in another study, a trend towards higher IC_{50} values was observed using J774.A.1 cells (mouse cell line) compared to peritoneal macrophages. It is unclear if such differences in activity were due to differences in host cells or inter-laboratory variability.*
- *In one study, miltefosine was shown to decrease viability but not permeability of cell membrane of promastigotes.*
- *The CC_{50} of miltefosine against macrophages was 37.8 µg/mL (92.7 µM). A ratio (SI) of cytotoxicity to biological activity (CC_{50}/IC_{50}) was variable (5 – 420 fold) (Table 8).*

Methods to measure in vitro sensitivity of Leishmania species to drugs are experimental methods and limited to research laboratories. Correlation of in vitro susceptibility of Leishmania species with clinical response has not been established.

Table 10: Activities of miltefosine against *Leishmania* species promastigotes and amastigotes

***Leishmania* species (strain)**	**IC_{50} µM (µg/mL)**	**Experimental conditions (incubation time and end point)***	**Reference**
Activity against promastigotes			
L. mexicana (MNYC/BZ/62/M379)	2.9 (1.2) at 24 hours 3.2 (1.3) at 48 hours	24 and 48 hours at 26°C; endpoint was proliferation.	Lux *et al*., 1996
L. mexicana (MNYC/BZ/62/M379 and MHOWBZ/84/BEL46)	14 (5.7)	72 hours at 28°C. Endpoint was viability by Alamar Blue	Lux *et al*., 2000
L. donovani (MHOM/SD/00/1S-2D)	10 (4.1)	14 hours at 25°C; viability measured by MTT assay	Luque-Ortega *et al*., 2007
L. donovani [MHOM/IN/54/LRC-L.51 (LRC-L.51)]	5.2 (2.1)	96 hours at 26°C. Endpoint number of promastigotes	Kuhlencord *et al*., 1992
L. donovani [MHOM/IN/80/DD8 (DD8)]	2.2 (0.9)		
L. infantum [MHOM/ES/86/STI-172 (STI-172)]	5.7 (2.3)		
L. donovani (MHOM/ET/67/L82)	0.36 - 0.45 (0.15 – 0.18)	10^6/mL for 72 hours at 26°C. Inhibition of promastigote growth was determined microscopically by counting parasite numbers	Escobar *et al*., 2002
L. major (MHOM/SA/85/JISH118)	4.8 – 13.1 (2.0-5.4)		
L. tropica (MHOM/AF/82/KOO1),	0.6 – 1.7 (0.25-0.70)		
L. aethiopica (MHOM/ET/84/KH),	1.2 – 2.8 (0.5-1.1)		
L. mexicana (MHOM/BZ/82/BEL21)	2.4 – 12.7 (0.98-5.2)		
L. panamensis (MHOM/PA/67/BOYNTON)	1.3 – 3.7 (0.53-1.5)		
L. donovani (MHOM/IN/54LRC-L.51)	5.1 (2.1)	96 hours at 26°C. Endpoint number of promastigotes	Unger *et al* , 1996
L. donovani [MHOM/IN/80/DD8 (DD8)]	25 (10.2)	48 hours at 24°C. Endpoint number of promastigotes	Verma and Dey, 2004
L. donovani [MHOM/ET/67HU3/L82]	13.6 (5.5)	10^6/mLfor 72 hours at 26°C. Inhibition of promastigote growth by MTT assay	Paris *et al*., 2004
L. donovani (clinical isolates) (n=24)	1.5–12.6 (0.6-5.1) 2.8-18.2 (1.1-7.4)	72 hours at 25°C and additional 24 hours after adding resazurin (Lab 1) 72 hours at 25°C and additional 4 hours after adding Alamar Blue (Lab 2)	Kulshrestha *et al*., 2013
Activity against amastigotes			
L. donovani (MHOM/ET/67/L82; LV9)	11.4 (4.7)	CD-1 - PEC incubated for 7 days at 37°C; % infected macrophages counted after Giemsa staining	Croft *et al*., 1987
L. donovani (MHOM/ET/67/L82)	3.9 (1.6)		Croft *et al*., 1996
L. donovani (MHOM/IN/82/Nandi II)	0.2 (0.08)		
L. donovani (strain not specified)	7.5 (3.1)	BALB/c and scid PEC; miltefosine added after 12 hours of infection and incubated for 3 days at 37°C; % infected macrophages counted after Giemsa staining	Escobar *et al*., 2001
L. donovani (MHOM/ET/67/L82)	3.3 – 4.6 (1.3-1.9)	CD-1 mice induced PEC infected with amastigotes in the ratio of 10:1 and incubated at 34°C for all species except *L. donovani* that were incubated at 37°C for 72 hours. The % infected macrophages counted after Giemsa staining	Escobar *et al*., 2002
L. major (MHOM/SA/85/JISH118)	31.6–37.2 (12.9-15.2)		
L. tropica (MHOM/AF/82/KOO1)	5.8 – 10.2 (2.4-4.2)		
L. aethiopica (MHOM/ET/84/KH)	2.6 – 4.9 (1.1-2.0)		
L. mexicana (MHOM/BZ/82/BEL21)	6.8 – 10.1 (2.8-4.1)		
L. panamensis (MHOM/PA/67/BOYNTON)	10.6 (4.3)		
L. amazonensis (IFLA/BR/1967/PH-8)	3.2 (1.3)	BALB/c mice - activated PEC infected with amastigotes in the ratio of 1:10. Miltefosine was added after 4 hours of culture and incubated for 72 hours at 37°C. The percentage of infected cells was counted after Giemsa staining. The results presented as the ratio of infection (number of amastigotes) between the treated and non-treated macrophage cultures.	Morais-Teixeira *et al*. 2011
L. brasiliensis (WHO-MHOM/BR/75/M2903)	5.4 (2.2)		
L. guyanensis (MHOM/BR/1997/321-P)	4.0 (1.6)		
L. chagasi (MHOM/BR/70/BH46)	4.5 (1.8)		
L. donovani (MHOM/ET/1967/HU3)	0.2 (0.09)		
L. donovani (clinical isolates) (n=28)	8.6 – 26.7 (3.5 – 10.9) IC_{90} values: 24.0 – 58.4 (9.8 – 23.8)	J774A.1 macrophage cell line infected in the ratio of 1:10 and miltefosine added after 24 hours of culture and incubated for 72 hours at 37 C. Intracellular amastigotes (infected macrophages) counted after Giemsa staining.	Prajapati *et al*., 2012
L. donovani (clinical isolates) (n=24)	0.3-24.9 (0.1-10.1) 0.03-6.13 (0.01-2.5)	48 hours at 37°C for J774A.1 cells (Lab 1) 48 hours at 37°C for mouse PEC (Lab 2	Kulshrestha *et al*., 2013

PEC=peritoneal exudate cells (macrophages); * there were other variabilities in experimental design such as medium used, number of passages of strains/isolates *in vitro* and/or animals (mice, hamsters)
Text in red represents clinical isolates

3.3. Activity *in vivo*

The activity of miltefosine was measured in murine models of visceral and cutaneous leishmaniasis.

3.3.1. Visceral leishmaniasis disease models

The activity of miltefosine was measured in mice infected with either *L. donovani* or *L. infantum*.

- ***L. donovani***

In a study by Croft *et al*. (1987),[18] the activity of miltefosine was reported in BALB/c mice infected by the intravenous route with freshly isolated amastigotes (5×10^6) of *L. donovani* (MHOM/ET/67/L82; LV9 strain). Treatment was initiated subcutaneously, a week later with miltefosine and other alkyl phosphorylcholines (100 mg/kg for 5 days) in one experiment and lower doses (45, 15 or 5 mg/kg for 5 days) in another experiment. Mice were sacrificed 4 days later and livers removed, weighed and impression smears prepared. The smears were fixed with methanol and stained with Giemsa stain. Drug activity was evaluated by comparing the number of amastigotes/500 liver cells in mice from untreated and treated groups. The results showed miltefosine at 100 mg/kg dose to be active in treating mice; however, miltefosine was toxic to mice at this dose level (Table 11). In a subsequent experiment the 50% effective dose (ED_{50}) of miltefosine was determined as 12.8 (14.3-11.4) mg/kg. At the highest dose tested (45 mg/kg) a 10% weight loss in mice following miltefosine treatment was recorded.

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Table 11: Activity of compounds against *L. donovani* in BALB/c mice at 100 mg/kg/day x 5 (s.c. route)

Compound	% inhibition (± standard error)	% Wt change of surviving mice after treatment*
I	100	−15.2†
III	98 ± 0.8	+1.9
VII	95 ± 1.7	−3.5
VIII	−1 ± 6.5	−1.2

* Untreated mice showed a +3.6% wt change over same time period.
† One mouse died on day 4 of treatment; surviving mice not treated on day 5.

Compound I = miltefosine

In another study (Croft *et al*., 1996[19]), the activity of miltefosine was reported against *L. donovani* strains MHOM/ET/67/L82 and MHOM/IN/82/Patna I, in mice. The experimental design was same as summarized above except that 10^7 amastigotes were used for infection and treatment was administered orally at doses between 3.75 and 30 mg/kg. Mice were sacrificed 3 days after the last dose. Livers and spleens removed, weighed and impression smears prepared from a cut surface for Giemsa staining. Drug activity was determined by comparing the number of amastigotes per 500 liver cells or spleen cells x organ weight (mg) in mice from treated and untreated groups. ED_{50} values were calculated by linear regression analysis. The results showed miltefosine was effective in reducing amastigotes in the liver (Table 12); the activity was about 3-fold higher (lower ED_{50} value) against the Indian strain compared to the Ethiopian strain; a similar trend in higher sensitivity (lower IC_{50} value) was observed *in vitro* against the Indian strain compared to the Ethiopian strain (Table 6).

Amastigote counts in spleen from treated and untreated mice were not shown.

Table 12: The activity of miltefosine against *L. donovani* in BALB/c mice

	% inhibition of amastigotes[a] in liver (dose in mg/kg) 30	15	7.5	3.75	ED_{50} values
Ilmofosine					
LV9 strain	67.3	35.4	28.7	53.9	14.5
Patna I strain	92.2	52.8	42.6	0	12.3
Miltefosine[b]					
LV9 strain	97.4	42.8	33.8	19.5	9.16
Patna I strain	98.9	97.4	88.8	44.3	2.9
Edelfosine[b]					
LV9 strain	22.9	23.7	48.2	24.2	—
Patna I strain	45.7	18.3	2.3	30.4	—
SR1 62-834					
LV9 strain	39.5	27.9	24.3	21.8	—
Patna I strain	74.9	18.9	15.1	7.7	—

[a]Oral administration, once/day for 5 days.
[b]Miltefosine is the generic name for hexadecylphophocholine (HDPC) and edelfosine is the generic name for ET-18-OCH_3.

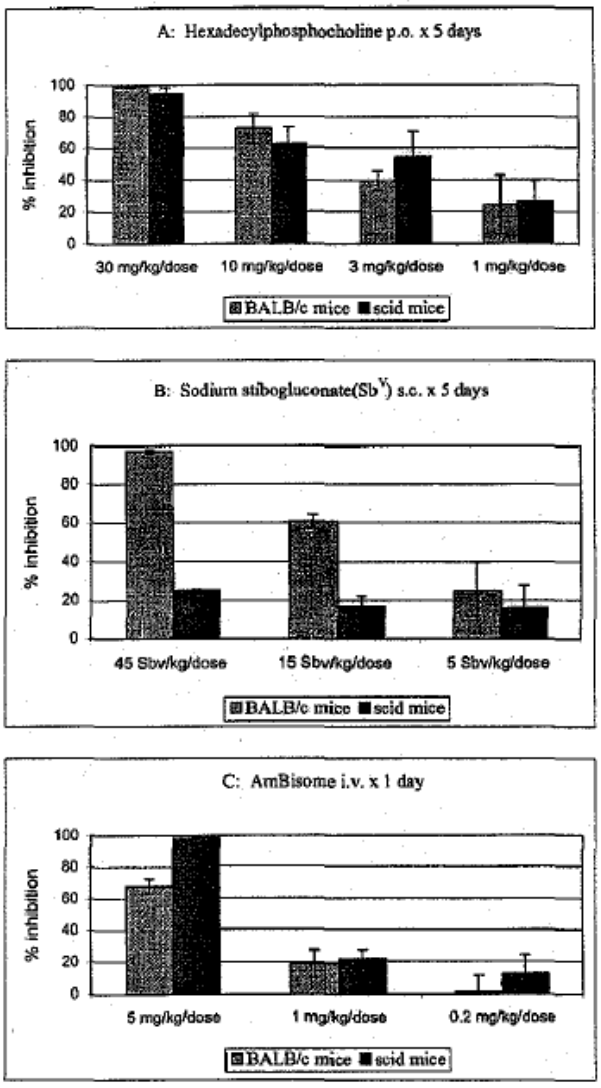
In another study (Escobar *et al*., 2001[20]), the activity of miltefosine was measured in CB-17 scid mice and BALB/c mice infected with the amastigotes (2 x 10^7) of *L. donovani* MHOM/ET/67/L82 strain. The experimental design was the same as summarized above except that treatment was initiated either 7 days or 14 days post-infection for 5 days. The Leishman Donovan Units (LDUs) in the liver were calculated as follows:

LDU= the number of amastigotes per 500 liver cells x organ weight (in milligrams)

The authors state that in an initial study, miltefosine at an oral daily dose of 30 mg/kg/dose for 5 days, was equally active in BALB/c and scid mice, with >95% parasite inhibition during the second week of infection (data not shown). Sodium stibogluconate at a daily dose of 45 mg/kg/dose for five days was significantly more active in BALB/c mice (87.35% ± 9.15% parasite inhibition) than in scid mice (41.00% ± 14.35% parasite inhibition).

The LDUs before treatment (2 weeks post-infection) were 1,804 ± 85 and 2,088 ± 78 in scid and BALB/c mice, respectively. At the end of the treatment (3 weeks after infection), the liver parasite burden was similar in both strains of untreated mice (LDUs of 2,335 ± 185 in scid mice and 2,375 ± 270 in BALB/c mice). Miltefosine showed similar dose-response effects in both BALB/c and scid mice (Figure 19A), with ED_{50}s and 90% effective dose (ED_{90}s) of 3.98 and 27.13 mg/kg/dose and 4.53 and 42.66 mg/kg/dose, respectively; similarly, the percentages of parasite inhibition by miltefosine at 30 mg/kg was comparable in both BALB/c and scid mice (98.68 and 94.7%, respectively). In contrast, sodium stibogluconate was more active in BALB/c mice than in scid mice with ED_{50} values of 20.26 and 56.53 mg/kg, respectively (Figure 19B); parasite inhibition was higher in BALB/c mice than in scid mice (96.26 and 28.8%, respectively). The drugs were well tolerated by the mice at the high doses tested, and no weight reductions were recorded in the mice in the treated groups. AmBisome at a single dose was active in both models (Figure 19C), with ED_{50}s and ED_{90}s of 2.91 and >5 mg/kg/dose and 1.51 and 3.1 mg/kg/dose in BALB/c and scid mice, respectively.

Figure 19: Effects of miltefosine (A), sodium stibogluconate (B), and AmBisome (C) against *L. donovani* amastigotes in the livers of BALB/c and scid mice.



Hexadecylphosphochline=miltefosine

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In another study (Kuhlencord *et al*., 1992[15]), activity of miltefosine was reported in female BALB/c mice infected with a mixture of promastigotes (10^7) and amastigotes (10^7) of 3 strains of *L. donovani* [MHOM/IN/54/LRC-L.51 (LRC-L.51) and MHOM/IN/80/DD8 (DD8) by the intravenous route. Treatment was initiated on day 7 post-infection. Miltefosine was administered orally at doses of 10 or 20 mg/kg/day for a week. Pentostam was administered subcutaneously at a dose of 120 mg/kg/day for 7 days or 4 weeks. The mice were killed on day 14, and parasite burden in the spleens and livers determined in Giemsa-stained impression smears from cut sections by counting the number of amastigotes per 1,000 spleen or liver cell nuclei. The total number of amastigotes per organ was calculated as follows:

Parasite burden per organ=organ weight (in milligrams) x number of amastigotes per nucleus X 200,000.

Parasite suppression (parasite burden in drug-treated mice to parasite burden in untreated mice) and parasite killing (parasite burden at the end of treatment to parasite burden at the start of treatment) were calculated for all strains on day 14 post-infection and for strain LRC-L.5l on days 21, 28, and 35 post-infection as well. The parasite burden (for mice infected with strain LRC-L.51) in the bone marrow was examined on days 7 and 35 post-infection. The femurs of

treated and untreated mice were removed, denuded and gently broken with forceps and the bone marrow carefully removed with a scalpel. Smear preparations were stained with Giemsa, and examined microscopically for Leishman-Donavan bodies.

To determine whether the microscopically detected parasites were viable or whether parasites were present even though they could not be detected in the stained impression smears, homogenates of spleen, liver, and bone marrow were cultured for a maximum of 3 weeks in HOSMEM-RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 26^{o}C. Cultures were examined daily for the presence of promastigotes.

At the time of treatment initiation on day 7, amastigotes were observed in liver and spleen in a subgroup of animals infected with the strain LRC-L.51 of *L. donovani*; there was no change in spleen weight (Table 13).

Table 13: Parasite burden at different time points post-infection in BALB/c mice[a]

Day after infection	Mean ± SD no. of leishmania (10^6) per:		Splenic wt (mg) (mean ± SD)
	Liver	Spleen	
0	0 ± 0	0.0 ± 0	90 ± 10
7	140 ± 20	1.8 ± 0.2	96 ± 10
14	579 ± 47	11.8 ± 2.8	130 ± 30
21	643 ± 32	17.4 ± 3.5	280 ± 10
28	538 ± 19	80.7 ± 12.4	600 ± 50
35	929 ± 90	120.6 ± 33.2	820 ± 30

[a] Female BALB/c mice (n = 3) were infected intravenously on day 0 with 10^7 *L. donovani* LRC-L.51 amastigotes and promastigotes. Liver and spleen impression smears were prepared on the indicated days. The number of amastigotes per 1,000 organ nuclei was counted under oil immersion, and the parasite burden was calculated as described by Stauber et al. (25).

Miltefosine treatment for 7 days was effective in reducing parasite burden in both liver and spleen of mice infected with either of the strains of *L. donovani* (Table 14); a dose of 20 mg/kg/day was more effective than the lower 10 mg/kg/day dose.

Table 14: Effect of treatment with miltefosine for 7 days on parasite burden in spleen and liver of mice infected with different strains of *L. donovani* (LRC-L.51 and DD8) and *L. infantum* (STI-172).

Parasite status, organ, and strain[a]	% Suppression or killing after the following treatment		
	120 mg of Sb^V/kg[b]	10 mg of He-PC/kg	20 mg of He-PC/kg
Parasite suppression[c]			
Liver			
LRC-L.51	97.2	91.7	97.8
DD8	96.9	93.6	99.9
STI-172	90.1	87.3	94.9
Spleen			
LRC-L.51	83.0	94.9	98.3
DD8	66.8	94.1	99.8
STI-172	18.3	70.7	75.3
Parasite killing[d]			
Liver			
LRC-L.51	88.3	65.2	90.9
DD8	90.4	80.4	99.5
STI-172	56.4	44.3	77.8
Spleen			
LRC-L.51	−11.1	66.7	88.9
DD8	−31.1	77.2	98.7
STI-172	−20.8	56.7	63.5

[a] Leishmania strains are described in detail in the text.
[b] Mice ($n = 3$) were given an injection of 0.2 ml of a pentostam solution (120 mg of Sb^V per kg) subcutaneously or 0.2 ml of an aqueous solution of He-PC (10 or 20 mg/kg) orally by gastric gavage once daily for 5 consecutive days. Organ impression smears were made 3 days after the end of treatment. The approximate amastigote count per liver and spleen was determined as described in footnote *a* of Table 1.
[c] Parasite suppression is the ratio of parasite load in drug-treated groups to the parasite load in untreated control groups. Organ impression smears were made 3 days after 5 days of treatment, and amastigote counts per organ were determined as described in footnote *a* of Table 1.
[d] Parasite killing is the ratio of parasite load in drug-treated groups after the end of treatment in comparison with the parasite load before the start of treatment.

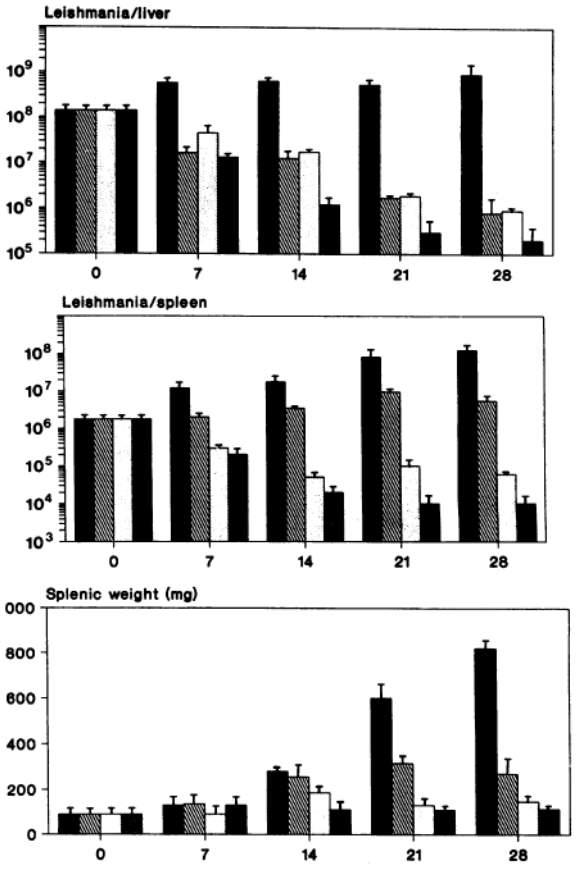
After 4 weeks of treatment there was a continuous decrease in the parasite load in the livers of mice infected with the LRC-L.51 strain of *L. donovani* and treated with miltefosine or Pentosam. In the spleen, miltefosine was more effective in reducing parasite burden compared to Pentostam. Oral treatment with 20 mg/kg of miltefosine resulted in more than a 3-log reduction of parasites and was 630-fold more effective than Pentostam. Overall, the results showed that the higher dose (20 mg/kg) of miltefosine was more effective (5- to 10-fold-higher) in suppressing the parasites than the lower dose (10 mg/kg) or the comparator, Pentosam (Figure 20). Spleen weights remained normal and were similar to uninfected control mice.

Bone marrow specimens from femurs were evaluated for parasites at the start (day 7) and at the end (day 35) of therapy. On day 7, 3 to 6 *Leishmania* parasites per 100 bone marrow cells were detected. After treatment with Pentostam, the parasite load on day 35 increased to 20 to 30 *Leishmania* parasites per 100 bone marrow cells. In contrast, miltefosine treatment led to a parasite suppression and killing; at a dose of 10 mg/kg dose of miltefosine, less than 1 parasite per 100 bone marrow cells were observed on day 35, whereas at a dose of 20 mg/kg, the number of parasites was below the detection limit.

Homogenates from the livers, spleens, and bone marrow of mice treated for 4 weeks with miltefosine or Pentostam were cultured to assess parasite growth. In Pentostam-treated mice,

cultures were positive within 3 days, cultures of bone marrow and spleens from miltefosine (20 mg/kg) treated mice were not positive before 8 days of incubation.

Figure 20: Effect of treatment with miltefosine for 4 weeks on amastigotes in spleen and liver parasite burden of mice infected with the LRC-L.51 strain of *L. donovani*



Bars represent standard errors of the means. ■, control; ▨ Pentostam; ▦ miltefosine (10 mg/kg); ▨ miltefosine (20 mg/kg).
Female BALB/c mice *(n* = 3) were infected intravenously with 10^7 amastigotes and promastigotes of the *Leishmania* strain LRC-L.51 on day -7. On day 0, treatment was begun with miltefosine (10 or 20 mg/kg) orally or 120 mg/kg of pentavalent antimony (Pentostam) subcutaneously. Drugs were given five times a week for up to 4 weeks.

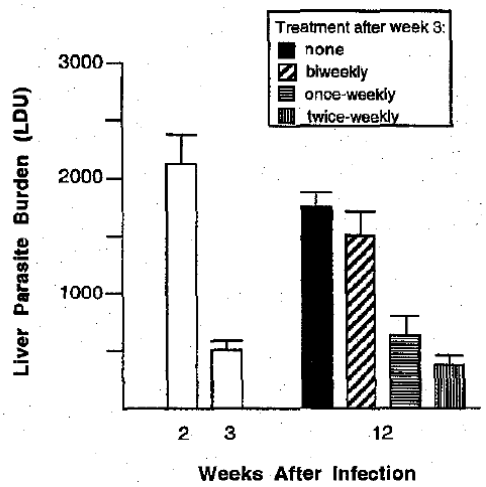
In another study (Murray, 2000[24]), the long term effect of treatment with miltefosine on prevention of recrudescence in T-cell deficient athymic (nude) BALB/c mice was reported. Mice were infected intravenously with 1.5 x 10^7 hamster spleen-derived *L. donovani* amastigotes (one Sudan strain). Visceral infection was monitored microscopically using Giemsa-stained liver imprints, and liver parasite burdens (expressed as LDU) were measured, in a blinded fashion, by calculating the number of amastigotes per 500 cell nuclei multiplied by the liver weight (in milligrams). The histologic reaction in the liver was assessed using formalin-fixed, stained tissue sections. Two weeks after *L. donovani* challenge, liver parasite burdens were determined for 4 of 28 infected mice, and then the animals received either no treatment *(*n = 4) or oral miltefosine by gavage (n = 20). Miltefosine was administered once daily at 25 mg/kg for 5 days. Two days after treatment ended, untreated (n=4) and treated (n=4) mice were sacrificed. LDU at this point (week 3) were compared to initial LDU (at week 2) to determine the extent of initial parasite

[24] Murray HW. Suppression of post treatment recurrence of experimental visceral leishmaniasis in T-cell-deficient mice by oral miltefosine. Antimicrob Agents Chemother (2000) 44: 3235-3236.

killing. The remaining 16 treated mice were randomly divided into 4 groups; for the next 9 weeks, the mice were administered either no further treatment or single doses of miltefosine (25 mg/kg) administered twice weekly, once weekly, or every 2 weeks. Twelve weeks after infection liver parasite burdens were determined for all animals.

Between week 2 and week 3, the period during which miltefosine was initially administered, liver parasite burdens increased in untreated nude mice from 2,124 ± 269 to 2,994 ± 212 LDU (mean ± the standard error of the mean [SEM]) (n = 8, data not shown). In mice treated for 5 days, LDU (mean ± SEM) at week 3 (i.e., 1 week after initiation of therapy) were reduced to 496 ± 87 (77% initial killing) (Figure 21). Treated mice were then administered either no additional miltefosine or single doses twice per week, once per week, or every second week for the next 9 weeks. The results showed that parasite replication resumed in mice that were administered no additional drug, and liver burdens at week 12 were 3.5-fold higher than those at week 3 (Figure 21) suggesting relapse. While miltefosine administered biweekly did not prevent recurrence of visceral replication, treatment given once or twice weekly was active in suppressing infection for the duration of the experiment. Histologic examination of the livers at week 12 confirmed these observations.

Figure 21: Initial effect of miltefosine treatment in nude-BALB/c mice and prevention of subsequent relapse.



Two weeks after infection, nude mice were initially treated with drug for 5 days. Two days later (end of week 3), treated mice either were given no further drug or received single doses every second week, once weekly, or twice weekly for the next 9 weeks. Results are from two experiments and are means ± SEMs for seven to eight mice per group.

In another study (Murray and Delph-Etienne, 2000[25]), the activity of miltefosine was measured in *L. donovani* (1 Sudan strain) infected mice deficient in T-cells, IFN-γ, and specific macrophage killing pathways. The objective was to learn whether endogenous immunologic mechanisms are required for or regulate the activity of miltefosine. Euthymic and athymic (nude) BALB/c mice and normal C57BL/6 and IFN-γ gene knockout (GKO) mice (bred on a C57BL/6 background), respiratory burst-deficient *gp91^{phox-1}* (X-linked chronic granulomatous disease [X-CGD]) mice and inducible nitric oxide (NO) synthase (iNOS) KO mice and their wild-type+/+ littermates (all on C57BL/6 X 129/Sv backgrounds) were included for testing. Mice (groups of 4

[25] Murray HW and Delph-Etienne S. Visceral leishmanicidal activity of hexadecylphosphocholine (miltefosine) in mice deficient in T cells and activated macrophage microbicidal mechanisms. J In Dis (2000) 181: 795-799.

– 6) were challenged via the tail vein with 1.5 x 10^7 hamster spleen-derived amastigotes. Visceral infection was followed microscopically by use of Giemsa-stained liver imprints, and liver parasite burdens determined by counting in a blinded fashion the number of LDU as summarized above. The histologic reaction in the liver was assessed by use of formalin fixed stained tissue sections. Granuloma formation at infected foci was scored as none, developing, or mature. Two weeks after infection (day 0), liver parasite burdens were determined, and mice were administered either no treatment or oral miltefosine by gavage.

There was no obvious toxicity, and 2 days after treatment ended (day + 7), all mice were killed and parasite burdens measured. Day +7 LDUs were compared with day 0 LDUs to determine percentage of parasite killing. The authors state that preliminary experiments in normal BALB/c mice showed that 5 days of treatment with 20, 25, and 30 mg/kg/day miltefosine induced 51%, 76%, and 81% mean reductions (killing) in liver parasite burdens, respectively, on day +7. A daily dose of 25 mg/kg was selected for further testing.

X-CGD mice were also treated continuously with aminoguanidine (2.5%) used as an iNOS inhibitor.

Response to treatment in T cell-deficient nude mice: In nude mice, a 63% reduction in liver parasite burden on day +7 was observed after treatment with miltefosine; such an effect was similar to that induced by treatment in normal BALB/c controls (Table 15).

Response to treatment in GKO mice: The response to miltefosine in IFN-γ-deficient mice was similar to that in C57BL/6 mice (72% killing vs. 82%, respectively; Table 15), suggesting no required cofactor role for endogenous IFN-γ or host defense mechanisms.

Response to treatment in mice lacking macrophage microbicidal mechanisms: The response to miltefosine in X-CGD or XCD + AG or iNOS KO mice was similar to C57BL/6 and wild-type mice (Table 15).

Role of tissue granuloma in the response to miltefosine: None of the 4 deficient hosts tested in this study showed a discernible granulomatous response at the time of treatment, suggesting that activity of miltefosine was granuloma independent. In normal C57BL/6 mice, the developing granulomatous response to *L. donovani* was rapid.

Overall, the study suggests that the activity of miltefosine was similar in immunocompetent and immunocompromised mice.

Table 15. Response to miltefosine in normal and immunodeficient mice.

Mice	Treatment	Liver parasite burden (LDU) Day 0	Liver parasite burden (LDU) Day +7	% killing[a]
BALB/c	None	1408 ± 142	1625 ±123	0
	MILT		391 ± 38	72
Nude BALB/c	None	2722 ± 205	3946 ± 323	0
	MILT		999 ± 104	63
C57BL/6	None	2163 ± 221	2766 ± 302	0
	MILT		375 ± 49	83
GKO	None	3264 ± 333	5225 ± 335	0
	MILT		928 ± 139	72
X-CGD	None	3399 ± 328	3291 ± 175	3
	MILT		515 ± 93	85
X-CGD + AG	None	4326 ± 318	3812 ± 214	12
	MILT		612 ± 107	86
Wild-type	None	2322 ± 204	2145 ± 197	8
	MILT		307 ± 45	87
iNOS KO	None	3306 ± 234	4092 ± 354	0
	MILT		248 ± 39	92

NOTE. Normal C57BL/6 mice served as controls for gene knockout (GKO) and X-linked chronic granulomatous disease (X-CGD) animals; wild-type littermates were controls for inducible nitric oxide synthase (iNOS) KO mice. Results from 2–3 experiments show mean ± SEM values for LDUs for 8–16 mice/group at time treatment was started (day 0, 2 weeks after infection) and 7 days later (day +7). LDU, Leishman-Donovan units; AG, aminoguanidine treatment.

[a] % killing = [(day 0 LDU − day +7 LDU)/day 0 LDU] × 100. In 4 groups of deficient mice, MILT-induced % killing on day +7 was not significantly different ($P > .05$) from that of treated control mice.

MILT= miltefosine

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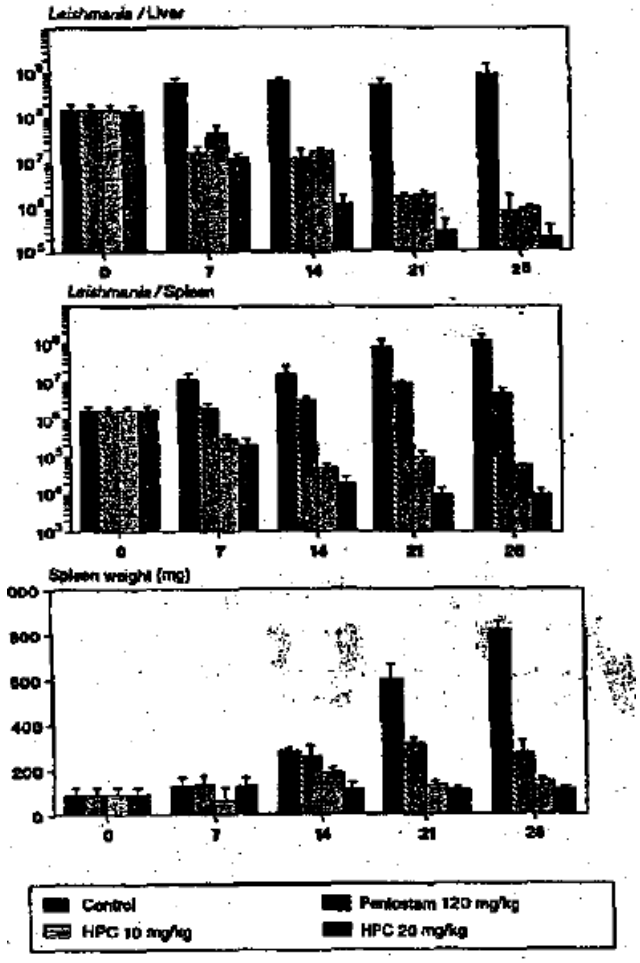
In another study (Unger *et al.*, 1998[16]), the activity of miltefosine was measured in female BALB/c mice infected intravenously with 10^7 promastigotes of the MHOM/IN/54LRC-L.51 strain of *L. donovani*. Treatment was initiated on day 7 at which time the mean parasitic burden was 100 to 200 x 10^8 amastigotes per liver, 1 to 2 x 1 0^6 per spleen, and 3 to 6 amastigotes per microscopic field of 100 hematopoietic cells in the bone marrow; duration of treatment was not specified. Parasite burden in spleen and liver was determined in Giemsa stained impression smears from cut sections by counting the number of amastigotes per 1,000 spleen or liver nuclei. The parasite burden per organ was calculated based on organ weight in milligrams x amastigotes per nucleus x 200,000. Parasite suppression (parasite burden of drug-treated mice to parasite burden of untreated mice) and parasite killing (parasite burden at the end of treatment to parasite burden at the start of treatment) were calculated on days 14, 21, 28 and 35 post-infection. The parasite burden in the bone marrow was examined on days 7 and 35·post-infection. Homogenates of spleen, liver and bone marrow were cultured for a maximum 3 weeks in HOSMEM, RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 26^{o}C. Cultures were examined daily for the presence of promastigotes. The results showed a reduction in parasite burden in the spleen and liver of infected mice compared to untreated mice (Table 16); however, the results of parasite burden at different time points were not shown.

In another experiment, the effect of a 4-week treatment with miltefosine was measured. The results showed miltefosine at a dose of 20 mg/kg was about 5- 10-fold more effective in suppressing parasites than the 10 mg/kg dose (Figure 22). Parasite load slightly increased during therapy with Pentostam, which corresponded to the development of splenomegaly over time.

Table 16: Parasite burden in liver and spleen of drug-treated mice.

Drug	Parasites (x 10^6) Liver	Spleen
Control	520	28
Pentostam	3.5	6.9
Compound 1	0.07	0.008
Compound 2	0.07	0.015
Compound 3	55	3.8
Compound 4	6	0.03
Compound 5	22	0.035
Compound 6	65	0.35
Compound 7	2.5	0.25
Compound 8	28	0.02
Compound 9	550	7.2

Figure 22: The effect of a 4-week treatment period on parasite burden in liver and spleen of *L. donovani* strain LRC-l51-infected BALB/c mice. The drugs were given at concentrations indicated above. Miltefosine (HPC) was administered orally, and Pentostam s.c. on a daily basis 5 days a week. Data represent the mean of 3 independent experiments; SD values were below 5%.



- ***L. infantum:***

In a study by Kuhlencord *et al*. (1992),[15] female BALB/c mice were infected with a mixture of promastigotes (10^7) and amastigotes (10^7) of *L. infantum* strain MHOM/ES/86/STI-172 (STI-

172) by the intravenous route. Experimental design was same as summarized above for *L. donovani* infection model. The results showed that miltefosine treatment for 7 days was effective in reducing parasite burden in both liver and spleen of mice (Table 14); a dose of 20 mg/kg/day was more effective than the lower 10 mg/kg/day dose.

In another study (Le Fichoux *et al*., 1998[26]) the activity of miltefosine was measured in BALB/c mice infected with *L. infantum* MON1 (MHOM/FR/94/LPN101) strain isolated from a patient with visceral leishmaniasis that was maintained by serial passages in Syrian hamsters. The promastigote forms were cultured *in vitro* and after 3 passages washed. Mice were infected intravenously with 10^8 promastigotes. Testing was performed under three different experimental conditions:

- Short-term efficacy against a recent infection.
- Short-term efficacy against an established infection.
- Long-term efficacy against an established infection.

Treatment was initiated orally on day 7, 42, or 28 for 5 days and mice sacrificed at different time points for evaluation of parasite burden in liver and spleen (Table 17). The amastigote burden was assessed by blinded microscopic enumeration with Giemsa-stained liver and spleen touch prints by two independent experienced parasitologists. The parasite load was expressed as LDU that was calculated as number of amastigotes per 1,000 nucleated cells × organ weight [in grams] × 2 × 10^5). The percent efficacy was calculated as [1 − (mean amastigote load in treated mice/mean amastigote load in untreated control i.e., NTC)] × 100.

Table 17: Experimental design - assessment of miltefosine activity against *L. infantum* infection in BALB/c mice[a]

Protocol	Treatment group (no. of mice)	Treatment period (dpi)[b]	Day(s) of amastigote burden evaluation (dpi)
1	HDPC (11) MEGAN (10) NTC (10)	D7–D11	D14
2	HDPC (10) MEGAN (10) NTC (10)	D42–D46	D49
3	HDPC (11)[c] NTC (11)[c]	D28–D32	D42, D63, D84 D42, D63, D84

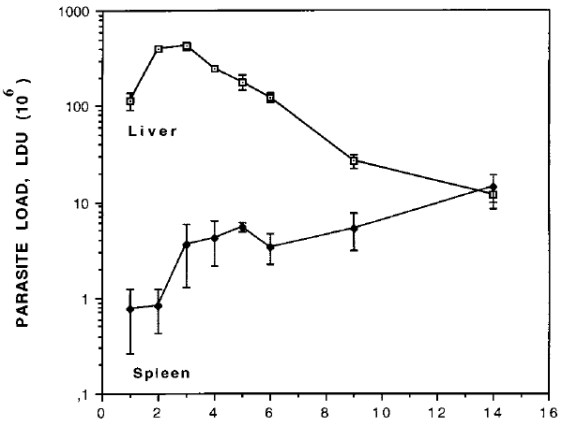
[a] Mice were intravenously inoculated with 10^8 stationary-phase *L. infantum* promastigotes/mouse. The day of infection is termed D0. Mice were randomly assigned to groups of 10 or 11 mice and were treated for 5 days with HDPC (20 mg/kg of body weight/day, oral administration) or MEGAN (200 mg/kg of body weight, subcutaneous injection) or were left untreated.
[b] dpi, day post infection.
[c] Three groups of 11 mice were used.

HDPC=miltefosine

At 6 weeks after promastigote inoculation, i.e., at the end of the acute phase of the disease, the parasite load in the liver decreased but was still high. The splenic parasite burden, one of the major sites of *Leishmania* multiplication in the natural infection, was initially low, but increased steadily for at least 3 months, and unlike the hepatic burden, it did not decline spontaneously without treatment (Figure 23).

[26] Le Fichoux Y, Rousseau D, Ferrua B, Ruette S, Lelievre A, Grousson D, and Kubar J. Short- and long-term efficacy of hexadecylphosphocholine against established *Leishmania infantum* infection in BALB/c mice. Antimicrob Agents Chemother. (1998) 42:654–658.

Figure 23: Course of *L. infantum* infection in BALB/c mice.



Mice were inoculated with 10^8 stationary-phase promastigotes/mouse via the caudal vein, and parasite loads were evaluated on liver and spleen touch prints at the indicated time points after injection. Visceral proliferation of amastigotes was quantified in LDU. Results are from a representative infection experiment, and the values are means ± SD for three mice at each time point. Patterns were similar in seven experiments.

Short-term efficacy against recent infection: Treatment was initiated on Day 7 for 5 days of daily dosing, and mice examined 3 days after the end of the treatment (Day14). The results showed no significant differences in the body, liver, and spleen weights between different groups. The mean [± standard deviation (SD)] liver amastigote burden (expressed in millions of LDU) on Day 14 was 158.9 ± 41.7 for the untreated control group (n = 10) compared to 0.13 ± 0.24 for the miltefosine treated group (n = 11) with amastigote suppression of 99.9%. The results for spleen parasite burden were not shown.

Short-term efficacy against established infection: When treatment was initiated on Day 42 for 5 days and mice evaluated 3 days after the last dose, activity was higher in the liver compared to spleen (Table 18). No wasting was detected during the course of the disease. The authors state that the degree of splenomegaly was notable, i.e., 3.3-fold normal spleen weight (which is on average 100 ± 10 mg), and there was no significant difference between the experimental groups, suggesting that 3 days after the end of miltefosine uptake, inflammation was still present in spite of the suppression of amastigotes.

Table 18: Short-term efficacy of miltefosine against established *L. infantum* infection in BALB/c mice[a]

Treatment group	Body wt (g)	Relative wt (%)[b]		Amastigote load, LDU (10^6) (% efficacy)[c]	
		Liver	Spleen	Liver	Spleen
HDPC	23.2 ± 0.8	5.6 ± 0.4	1.4 ± 0.2	8.8 ± 9.6 (93.8)	2.2 ± 1.8 (77.9)
MEGAN	24.2 ± 0.9	5.8 ± 0.3	1.4 ± 0.2	21.7 ± 20.5 (84.8)	4.5 ± 2.3 (54.7)
NTC	24.6 ± 0.6	6.0 ± 0.5	1.4 ± 0.2	142.2 ± 75.7	9.8 ± 6.0

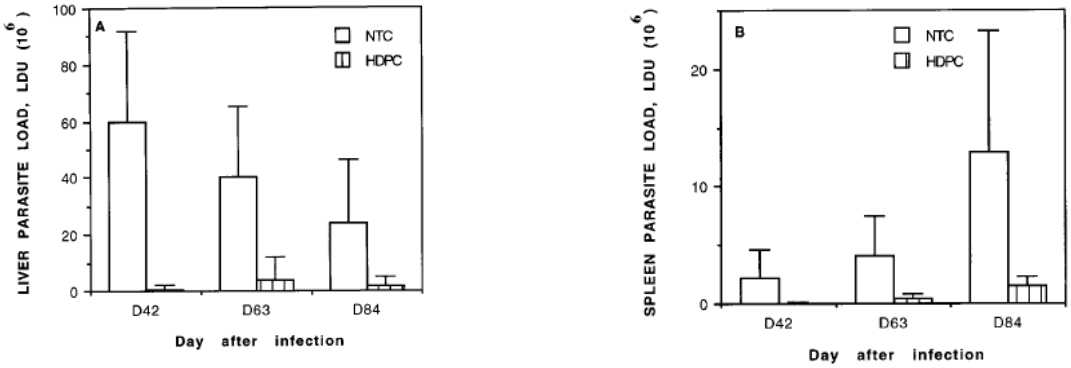
[a] Mice, intravenously inoculated with 10^8 stationary-phase *L. infantum* promastigotes/mouse on D0, were treated for 5 days, starting D42, with HDPC (20 mg/kg of body weight/day, oral administration) or MEGAN (200 mg/kg of body weight/day, subcutaneous injection) or were left untreated. Mice were examined 3 days later (D49). Data are means ± SD for 10 mice in each group.
[b] Organ weight/body weight.
[c] LDU, number of amastigotes per 1,000 nucleated cells × organ weight (in grams) × 2 × 10^5. Percent efficacy, [1 − (mean amastigote load in treated mice/mean amastigote load in control mice)] × 100.

Course of *L. infantum* infection in BALB/c mice. Mice were inoculated with 10^8 stationary-phase promastigotes/mouse via the caudal vein, and parasite loads were evaluated on liver and spleen touch prints at the indicated time points after injection. Visceral proliferation of amastigotes was quantified in LDU. Results are from a representative infection experiment, and the values are means ± SD for three mice at each time point. Patterns were similar in seven experiments.
HDPC=hexadecyl phosphocholine = miltefosine

Long-term efficacy against established infection: Treatment was initiated on Day 28 for 5 days and mice evaluated after prolonged time periods (Day 42, 63, and 84 i.e., 10, 31, and 52 days after the last dose). The parasite burden in infected untreated mice decreased in the liver and increased steadily in the spleen between Days 42 and 84. In miltefosine treated mice, the hepatic

and splenic amastigotes (Figure 24) were suppressed by 98.8% on Day 42 and remained significantly lower (around 90% suppression) than the untreated controls throughout the duration of the experiment. Splenomegaly was apparent and much more in the untreated mice group compared to miltefosine treated mice.

Figure 24: Long-term efficacy of miltefosine against full-blown *L. infantum* infection in BALB/c mice.



Mice, intravenously inoculated with 10^8 stationary-phase promastigotes/mouse on Day 0, were treated orally for 5 days, starting on Day 28, with miltefosine (20 mg/kg/day) or were left untreated. The effects on amastigote burden in the liver (A) and spleen (B) were examined 10, 31, and 52 days after the end of the treatment (Day 42, Day 63, and Day 84, respectively). Results were expressed in LDU, and values are means ± SD for 11 mice in each group and at each time point. NTC= Untreated control. HDPC=miltefosine

Treatment group	Day of evaluation	Body wt (g)	Relative wt (%)[b]		% Efficacy[c]	
			Liver	Spleen	Liver	Spleen
HDPC	D42	23.2 ± 1.0	4.7 ± 0.6	0.5 ± 0.1	98.9	98.8
	D63	22.4 ± 0.7	5.6 ± 0.4	0.6 ± 0.1	90.4	91.8
	D84	25.4 ± 0.6	4.9 ± 0.4	0.6 ± 0.1	92.5	88.8
NTC	D42	23.5 ± 1.0	5.2 ± 0.4	0.8 ± 0.2		
	D63	22.1 ± 0.7	6.1 ± 0.4	1.3 ± 0.3		
	D84	25.0 ± 0.5	5.4 ± 0.5	2.0 ± 0.4		

[a] Mice, intravenously inoculated with 10^8 stationary-phase *L. infantum* promastigotes/mouse on D0 and treated for 5 days (starting D28) with HDPC (20 mg/kg of body weight/day, oral administration) or left untreated, were examined on D42, D63, and D84 (10, 31, and 52 days, respectively, after the end of the treatment). Values are means ± SD for 10 mice per group.
[b] Organ weight/body weight.
[c] [1 − (mean amastigote load in treated mice/mean amastigote load in NTC)] × 100. The parasite load data for the HDPC-treated and control mice are shown in Fig. 2.

Comments:

- *The studies show miltefosine to be effective in mice infected with L. donovani.*
  - *Miltefosine was effective in both acute and chronic infection models in reducing parasite burden in liver and/or spleen.*
  - *In both scid and BALB/c mice L. donovani models, miltefosine and AmBisome had similar levels of activity. In contrast, sodium stibogluconate (Pentostam) was less active against L. donovani in scid mice than in BALB/c mice. These observations support the findings of similar miltefosine IC_{50} values against the amastigotes when tested with peritoneal macrophages derived from either scid or BALB/c mice.*

- *The activity of miltefosine in T cell-deficient nude mice, GKO mice, mice lacking macrophage microbicidal mechanisms (X-CGD or XCD + AG or iNOS KO) infected with L. donovani was similar to normal C57BL/6 or BALB/c mice.*
- *In one study, the activity of miltefosine was about 3-fold lower higher the Indian strain of L. donovani compared to the Ethiopian strain; a similar trend in vitro sensitivity was observed in vitro (Table 6).*
- *In nude BALB/c mice, one week of miltefosine treatment was effective in suppressing parasites but did not prevent recurrence. However, long term treatment (once or two times a week for 9 weeks) was effective in preventing relapse/recurrence.*

- *In mice infected with L. infantum miltefosine was effective in both acute and established visceral leishmaniasis disease models.*

3.3.2. Cutaneous leishmaniasis disease model

Schmidt-Ott *et al*. (1999),[27] reported the effect of topical treatment with miltefosine in a murine cutaneous leishmaniasis model. BALB/c, CBA/J and C57BL/6 mice were infected subcutaneously with 10^7 amastigotes of *L. mexicana* [strain MNYC/BZ/62/M379 (New world species)]. C57BL/6 mice were infected subcutaneously at the tail base with 2 x 10^6 *L. major* promastigotes [MRHO/IR/76/vaccine strain (Old world species)]. Treatment of the established lesions was initiated 5 months later for *L. mexicana* infected mice or 3 weeks later for *L.* major infected mice by applying one drop of Miltex® (about 1.5 mg miltefosine) daily with a small brush directly on to the lesions for 5 days a week, for 5 weeks *(L. mexicana* infected CBA/J and C57BL/6 mice) or 2 weeks *(L. mexicana* infected BALB/c mice and *L. major* infected C57BL/6 mice) (Table 19). The parasite burden in draining lymph nodes (inguinal and lower periaortic) and spleen was assessed in groups of at least 3 mice per strain at the onset of therapy and 3 weeks after the end of treatment *(L. mexicana* infected mice) or immediately after the end of treatment *(L. major*-infected mice). Briefly, the lymph nodes and spleens were cut into small pieces, single-cell suspensions prepared, and passed through a fine wire mesh and counted. Cell suspensions were serially diluted in quadruplicate in half-logarithmic steps in flat-bottom microtiter plates and incubated at 27°C. After 4-5 days, cultures were inspected for the presence of flagellated parasites (promastigotes). The total number of parasites per organ was estimated. The presence of persistent parasites at the original lesion site was determined by examining an aliquot of the scar tissue homogenate for the presence of flagellated parasites after 4 days of culture at 27°C.

The results showed that subcutaneous infection of BALB/c, CBA/J and C57BL/6 mice with 10^7 *L. mexicana* amastigotes led to large nodular lesions after 5 months. In all *L. mexicana*-infected mice visible and palpable lesions disappeared after the second week of treatment. In BALB/c mice treatment was then stopped, but was continued for 5 weeks in CBA/J and C57BL/6 mice. In the scars from 6 of 14 mice (3 BALB/ c and 3 CBA/J mice) parasites could be detected 3 weeks after the end of treatment.

At the onset of treatment, mean parasite numbers in draining lymph nodes from BALB/c, CBA/J and C57BL/6 mice were 7.3 x 10^5, 10^5, and 2.1 x 10^4, respectively; in spleen, the parasite counts were 7900, 650 and 1200, respectively (Table 19). The parasite count was decreased or absent at

[27] Schmidt-Ott R, Klenner T, Overath P, and Aebischer T. Topical treatment with hexadecylphosphocholine (Miltex®) efficiently reduces parasite burden in experimental cutaneous leishmaniasis. Trans Roy Soc Trop Med Hyg (1999) 93: 85-90.

the end of treatment in both the draining lymph nodes and spleen (Table18). Persistent parasites could be detected in the scar tissue of 3 of 6 BALB /c mice and 3 of 3 CBA/J mice but not in the 5 C57BL/6 mice (detection limit: 2 parasites per homogenized scar).

In C57BL/6 mice infected with 2 x 10^6 *L. major* promastigotes small nodular lesions were observed after 3 weeks. At the onset of treatment the mean parasite number in draining lymph nodes was 9000; the mean parasite number in draining lymph nodes in untreated mice was about1000-fold higher than in miltefosine treated mice (Table 19). No palpable lesions were detected in miltefosine treated mice whereas they were still palpable in untreated mice. No parasites could be detected in the spleen in any mouse (detection limit: 100 parasites per spleen).

The authors state that BALB/c, CBA/J and C57BL/6 mice infected with *L. mexicana* developed progressively growing lesions. Susceptibility of these mouse strains, as estimated by the kinetics of lesion growth, correlated with the parasite burden in draining lymph nodes (BALB/c > CBA/J > C57BL/6). It is unclear whether such differences in susceptibility of different mice strains are due to the differences in inoculum concentrations or host susceptibility.

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Table 19: Experimental design and estimation of parasite number

A. Experimental design

***Leishmania* species**	**Mouse strain**	**Amastigote inoculum concentration**	**Parasite count at the time of treatment initiation**		**Time of initiation (Duration) of treatment**
			Lymph nodes*	**Spleen**	
L. mexicana	BALB/c	10^7	7.3×10^5	7900	5 months (5 days/wk x 2 wks)
	CBA/J	10^7	10^5	650	5 months (5 days/wk x 5 wks)
	C57BL/6	10^7	0.21×10^5	1200	5 months (5 days/wk x 5 wks)
L. major	C57BL/6	2×10^6	9000	Not detected	3 weeks (5 days/wk x 2 wks)

*Draining lymph nodes

B. Effect on parasite burden in *L. mexicana* infected mice

	Pre-treatment[a]		Post-treatment[b]	
Mouse strain	Draining lymph nodes[c]	Spleen	Draining lymph nodes[c]	Spleen
BALB/c	410 000	22 000	<100	<100
	1 100 000	42 000	<100	<100
	700 000	550	<100	<100
CBA/J	130 000	650	100	<100
	70 000	700	100	100
			<100	<100
C57BL/6	24 000	950	<100	<100
	52 000	1150	<100	<100
	8000	1600	<100	<100

Mice were infected subcutaneously with 10^7 *L. mexicana* amastigotes at the tail base. Five months post infection established lesions were treated for 5 weeks (CBA/J and C57BL/6 mice) or 2 weeks (BALB/c) by topical application of Miltex®. The total parasite number in draining lymph nodes and spleen in individual mice was estimated by culturing single-cell suspensions in serial dilution.
[a]At onset of treatment, i.e. 5 months post infection.
[b]3 weeks after end of treatment.
[c]Inguinal and lower peri-aortic lymph nodes.

C. Effect on parasite burden in *L. major* infected mice

Pre-treatment[b]	Not treated[c]	Post-treatment[c]
26 800	59 200	30
770	2000	15
780	3600	50
7700	53 000	10

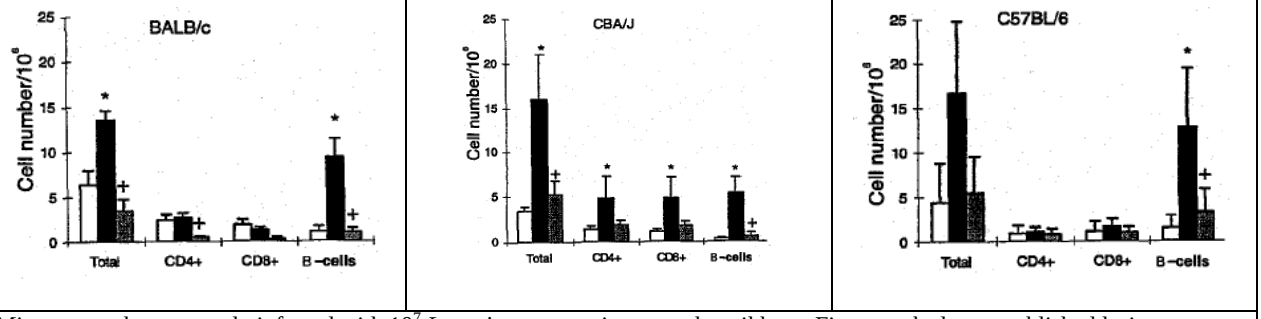
C57BL/6 mice were infected subcutaneously with 2×10^6 *L. major* promastigotes at the tail base. Three weeks post infection established lesions were treated for 2 weeks by topical application of Miltex®. The total parasite number in draining lymph nodes of individual mice was estimated by culturing single-cell suspensions in serial dilution.
[a]Inguinal and lower peri-aortic lymph nodes.
[b]3 weeks post infection.
[c]5 weeks post infection.

Cellular composition of draining lymph nodes:

In *L. mexicana* infected mice the lymphocytes of the draining lymph nodes were phenotyped for CD4, CD8 and B-cells by flow cytometry. The results showed that *L. mexicana* infection was associated with alteration of lymph node cellular composition in all the 3 mouse strains investigated (Figure 25); 5 months after infection, there was an increase in B-cell number in the draining lymph nodes. $CD4^+$ and $CD8^+$ T-cell numbers remained unchanged with the exception of a 3-fold increase in CBA/J mice. After miltefosine treatment cell counts were reduced to the

values found in healthy mice, with the exception of T -cell numbers ($CD4^+$ and $CD8^+$) in BALB/c mice that were lower than in healthy control mice.

Figure 25: Cellular content in draining lymph nodes

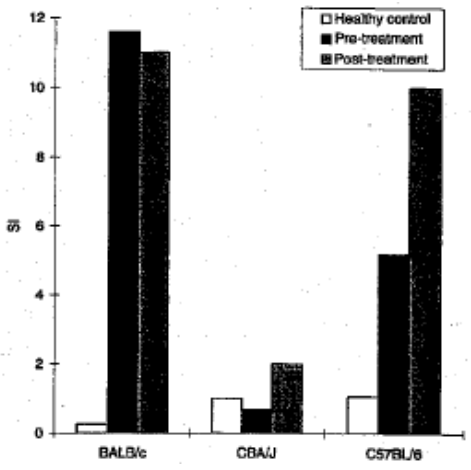


Mice were subcutaneously infected with 10^7 *L mexicana a*mastigotes at the tail base. Five months later established lesions were treated as described in Table 18A. Single-cell suspensions of draining lymph nodes of 3 mice per strain were analyzed. White bars: syngeneic healthy control mice; black bars: pre-treatment, i.e., 5 months post infection; grey bars: post-treatment, i.e. 3 weeks after end of treatment.
* Significantly different from syngeneic healthy control mice (t-test, $P < 0{\cdot}05$).
+ Significantly different from pre-treatment values (t-test, *P<0·05).*

The functional phenotype of $CD4^+$ lymph node cells:

Five months after infection with *L. mexicana*, $CD4^+$ cells from the draining lymph nodes were enriched and isolated and their proliferation and cytokine (IL-4 and IFN-γ) release measured *ex vivo* in response to heat-killed amastigotes (final concentration 10^6/mL) or freeze-thaw lysate of promastigotes (final concentration equivalent to 10^6 /mL). Testing was performed in the presence of 10 irradiated (2500 rad) syngeneic spleen cells from healthy donor mice as antigen-presenting cells (APC) at various effector cell/APC ratios (1:1, 1:3, 1:10). Concanavalin A (Con-A; final concentration 2.5 µg/mL), a nonspecific stimulator for T-cells, was used as a positive control. Cells from BALB/c and C57BL/6 mice incorporated 12 and 17 times more ^{3}H-thymidine than unstimulated cells after stimulation with heat-killed amastigotes; cells from CBA/J mice did not respond (Figure 26). The stimulation of cells from uninfected BALB/c, CBA/J or C57BL/6 mice in response to heat-killed *L. mexicana* amastigotes was same as that of unstimulated cells (Figure 26). $CD4^+$ lymph node cells of all mouse strains produced no detectable IL-4 (threshold of detection 1U /mL) or IFN-γ (threshold of detection 0.1 U/mL) following *Leishmania*-specific stimulation. However, lymphokine production was detected in cells from healthy, infected, or treated mice stimulated with Con-A suggesting specificity of response to *Leishmania* antigen. Topical miltefosine treatment did not alter response to *Leishmania* antigens or ConA.

Figure 26: *Leishmania* specific proliferation of purified $CD4^+$ cells from draining lymph nodes before and after topical treatment with miltefosine.



Mice were subcutaneously infected with 10^7 *L. mexicana* amastigotes at the tail base. Five months later established lesions were treated. Single-cell suspensions from inguinal and lower peri-aortic lymph nodes of 3 mice per mouse strain and time point were pooled and $CD4^+$ cells negatively selected by MACS. Cells (10^5) were co-incubated with 10^5 irradiated (2500 rad) naive spleen cells and stimulated with heat-treated *L. mexicana* axenic amastigotes (10^6 /mL). Cells were pulsed overnight with ^{3}H-thymidine. ^{3}H incorporation was measured by liquid scintillation counting on Day 3. The results expressed as specific stimulation index (SI). The background c.p.m. values (syngeneic healthy mice/mice before treatment/ mice after treatment) of cells from *BAIB/c*, CBA/J and C57BL/6 mice were 400/1000/950, 2000/10300/2000 and 2000/8800/1500, respectively.

Comments:

- *Overall, the study showed that topical treatment with miltefosine was effective in reducing parasite burden and lesions in all mice infected with L. mexicana or C57B/6 mice infected with L. major. Although miltefosine was effective in all the 3 strains of mice infected with L. mexicana, parasites were still detectable in the draining lymph nodes from CBA/J mice suggesting variability in treatment effect. This may be associated with host immune response as antigen-specific lympho-proliferation was not observed in CBA/J mice but was observed in BALB/c and C57BL/6 mice.*
- *The effect of oral treatment in the cutaneous leishmaniasis disease model was not measured.*

3.4. Drug Resistance and Cross Resistance

3.4.1. Drug Resistance

3.4.1.1. In vitro

- ***Potential for development of drug resistance***

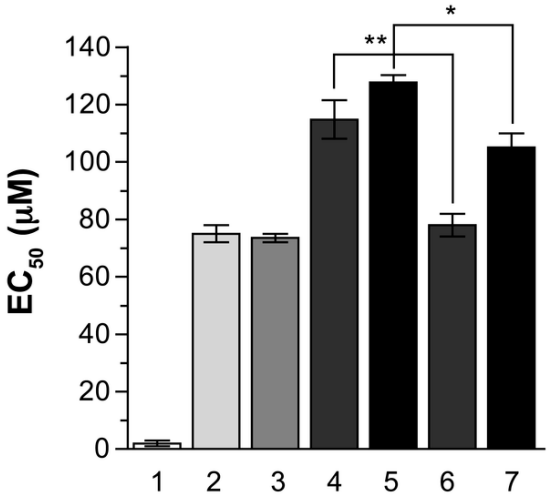
L. donovani promastigote clones resistant to miltefosine were generated *in vitro* by serial passage and step-wise increase in drug concentration (2.5 to 40 µM i.e., 1.0 to 16.3 µg/mL).[28] Briefly, the wild type promastigote clone was exposed to 2.5 µM i.e., 1µg/mL miltefosine for ten passages over 8 weeks before growth of the wild type clone and resistant (M-2.5R) line was equivalent. This was followed by incubation of the M-2.5R line with 5.0 µM (i.e., 2.0 µg/mL) miltefosine for eight passages over 5 weeks; the M-5R line was then incubated with 10 µM (i.e., 4.1 µg/mL) miltefosine for four passages over 3 weeks and M-10R line to 20 µM (i.e., 8.2 µg/mL) miltefosine for four passages over 4 weeks and M-20R line to 40 µM (i.e., 16.3 µg/mL) miltefosine for five passages over 4 weeks. The clone selected at 40 µM (16.3 µg/mL) miltefosine concentration was 15 times less susceptible to the drug (IC_{50} value 77.5 µM i.e., 31.6

[28]Seifert K, Matu S, Javier Perez-Victoria F, Castanys S, Gamarro F, and Croft S. Characterisation of *Leishmania donovani* promastigotes resistant to hexadecylphosphocholine (miltefosine). Int J Antimicrob Agents (2003) 22: 380–387.

µg/mL) than the wild-type clone (IC_{50} 5.1 µM i.e., 2.1 µg/mL) and showed cross-resistance to another alkylphospholipid (edelfosine) but not to the standard anti-leishmanial drugs. Resistance was stable up to 12 weeks in drug-free culture medium. No amplification of specific genes, including the multidrug resistance P-glycoprotein gene, was detected in the resistant parasites. Three attempts to increase the level of resistance to 80 µM (i.e., 32.6 µg/mL) miltefosine concentration were unsuccessful as the M-40R line promastigotes were unable to adapt at this concentration.

Similar observations were reported for *L. major* Friedlin strain (Figure 27).[29]

Figure 27: Miltefosine susceptibilities of *L. major* mutants and wild-type parasites.



The IC_{50} (EC_{50}) values were determined by culturing promastigote parasites in the presence of increasing concentrations of miltefosine. The average of three independent experiments is shown.
IC_{50} values for miltefosine against *L. major*
(1) wild-type parasites;
(2) MF80.1 MF-resistant mutants
(3) MF80.2 MF-resistant mutants
(4) MF80.3 MF-resistant mutants
(5) MF80.5 MF-resistant mutants
(6) MF80.3 mutants grown in the absence of MF for 30 passages, and
(7) MF80.5 grown in the absence of MF for 30 passages.
* $p<0.05$; ** $p<0.01$.
MF=miltefosine

A study by Moreira *et al.* (2011)[13] showed that *L. infantum* mutants resistant to miltefosine, sodium stibogluconate, or amphotericin B not only failed to undergo apoptosis following exposure to their respective drugs, but also were more tolerant towards apoptosis induced by other anti-leishmanial drugs, provided that the drug (such as pentamidine) killed *Leishmania* via reactive oxygen species production. Overall, the study suggests an acquisition of resistance. The study also suggests cross-resistance with drugs that have similar mechanism of action; however, no such drug is currently approved for the treatment of leishmaniasis.

- ***Mechanisms of drug resistance***

Mechanisms of resistance have been reported using different *Leishmania* species.

Studies with L. donovani

An increase in IC_{50} value for the resistant clone of *L. donovani* was shown to be associated with a defect in translocation of miltefosine across the plasma cell membrane.[30] Such an effect

[29] Coelho AC, Boisvert S, Mukherjee A, Leprohon P, Corbeil J, and Ouellette M. Multiple mutations in heterogeneous miltefosine-resistant *Leishmania major* population as determined by whole genome sequencing. PLoS Negl Trop Dis (2012) 6: e1512.

[30] Perez-Victoria FJ, Castanys S, and Gamarro F. *Leishmania donovani* resistance to miltefosine involves a defective inward translocation of the drug. Antimicrob Agents Chemother (2003) 47: 2397–2403.

appears to be related to a defect in drug internalization (i.e., transport over the parasite membrane) into the parasite as possible mechanisms of resistance.[30, 31, 32]

Miltefosine internalization in *L. donovani* is thought to require at least two proteins in the plasma membrane, *L. donovani* miltefosine transporter (LdMT), a P-type ATPase involved in phospholipid translocation, and its β subunit, LdRos.[30, 31, 32] The miltefosine transporter and the protein together play an important role in maintaining the phospholipid asymmetry of the parasite membrane.[33]

A miltefosine-resistant strain of *L. donovani* showed changes in the length and level of unsaturation of fatty acids, as well as a reduction in ergosterol levels suggesting that fatty-acid and sterol metabolism are probably targets for miltefosine resistance (Rakotomanga *et al*., 2005 and 2007).[7, 8]

Studies with L. braziliensis
Low expression of miltefosine transporter and protein complex were shown to correlate with intrinsic resistance to miltefosine of *L. braziliensis* strains.[34] The *L. brasiliensis* strains included for testing were

- Brazilian isolate MHOM/BR/75/M-2904 (WHO reference strain), and
- Peruvian isolates MHOM/PE/03/LH-2419, MHOM/PE/02/LH-2210, and MHOM/PE/03/LH-2224).

L. donovani MHOM/ET/67/HU3 (WHO reference strain) and derivative lines *LdMT* knockout (*LdMT*$^{-/-}$), *LdRos3* knockout (*LdRos3*$^{-/-}$), and *LdMT*$^{-/-}$ overexpressing LdMT-green fluorescent protein (GFP) were included for comparison.

The results showed higher IC_{50} values for *L. braziliensis* strains compared to *L. donovani* (Figure 28A); lower *in vitro* sensitivity of *L. brasiliensis* appeared to be associated with reduction in the ability to internalize the drug from the extracellular medium and not efflux of the drug (Figure 28 B to D). Such an effect was mainly due to the low expression levels of the miltefosine translocation machinery at the parasite plasma membrane using polyclonal antibodies against recombinant polypeptides for LdMT and LdRos3; these antibodies were cross-recognized by *L. braziliensis* miltefosine transporter (LbMT) and LbRos3. Different crude membrane preparations from different *L. braziliensis* strains showed a decrease in LbMT expression of about 90%

[31] Perez-Victoria FJ, Gamarro F, Ouellette M, and Castanys S. Functional cloning of the miltefosine transporter. A novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. J Biol Chem (2003) 278: 49965–49971.

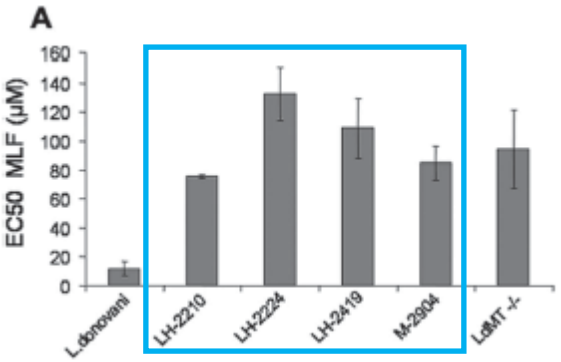
[32] Perez-Victoria FJ, Sanchez-Canete MP, Castanys S, and Gamarro F. Phospholipid translocation and miltefosine potency require both *L. donovani* miltefosine transporter and the new protein LdRos3 in *Leishmania* parasites. J Biol Chem (2006) 281: 23766–23775.

[33]Weingartner A, Drobot B, Herrmann A, Sanchez-Canete MP, Gamarro F, Castaanys S, and Pomorski TG. Disruption of the lipid-transporting LdMT-LdRos3 complex in *Leishmania donovani* affects membrane lipid asymmetry but not host cell invasion. PLoS ONE (2010) 5: e12443.

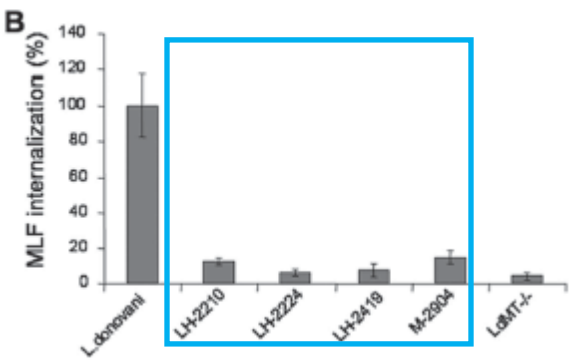
[34] Sanchez-Canete MP, Carvalho L, Perez-Victoria FJ, Gamarro F, and Castanys S. Low plasma membrane expression of the miltefosine transport complex renders *Leishmania braziliensis* refractory to the drug. Antimicrob Agents Chemother (2009) 53: 1305–1313.

compared to LdMT from wild-type *L. donovani* suggesting low level of miltefosine internalization by *L. braziliensis* strains included for testing compared to *L. donovani*.

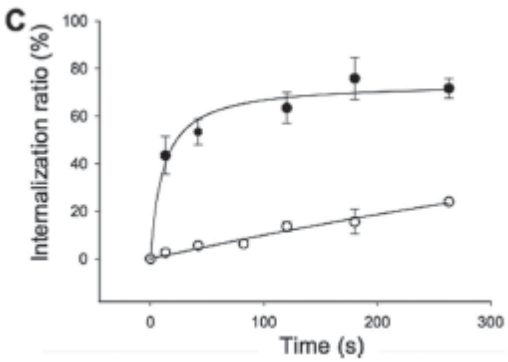
Figure 28: *L. braziliensis* strains are less sensitive to miltefosine (A) and have less drug internalization (B and C) than *L. donovani*. Time-dependent miltefosine efflux was similar in both species (D)



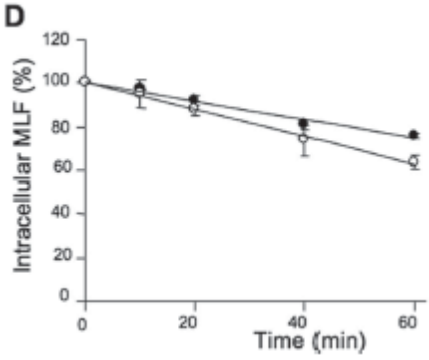
Miltefosine sensitivity of promastigotes of the *L. donovani* wild-type and *LdMT*$^{-/-}$ lines and *L. braziliensis* strains LH-2210, LH-2224, LH-2419, and M-2904. Shown are IC_{50}s after 72 hours of culture.



^{14}C-miltefosine internalization into promastigotes of the *L. donovani* wild-type and *LdMT*$^{-/-}$ lines and *L. braziliensis* strains LH-2210, LH-2224, LH-2419, and M-2904. Internalization was measured after 60 minutes of incubation at 28°C and expressed as a percentage of the internalization by wild-type *L. donovani*.



Initial rate of ^{14}C- miltefosine internalization in promastigotes of the *L. donovani* wild-type (black circles) and *L. braziliensis* LH-2419 (open circles) strains. The data are expressed as the percentage of ^{14}C-miltefosine inside the cells with respect to the drug bound to the outer leaflet of the plasma membrane at the indicated time points.

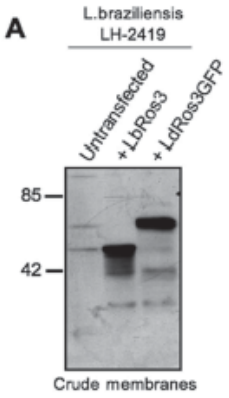


^{14}C- miltefosine efflux. Promastigotes of the *L. donovani* wild-type (black circles) and *L. brasiliensis* LH-2419 (white circles) strains were pre-incubated with ^{14}C-miltefosine at 28°C, and the decay in radioactivity was monitored at different times (10, 20, 40, and 60 minutes). The data are expressed as the percentage of the initial amount of ^{14}C-miltefosine incorporated.

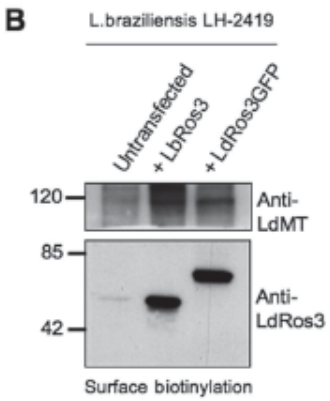
Bars or each point represent the mean ± standard deviation of three independent experiments.
MLF=miltefosine; EC_{50}=IC_{50}

Transfected *L. braziliensis* parasites with constructs containing LbRos3 and LdRos3-GFP showed an increase in LbMT levels at the plasma membrane compared to the parental line. Miltefosine internalization was similarly increased around five-fold in each transfected line and the sensitivity to miltefosine was also increased three- to four-fold (Figure 29). Overall, the studies showed that overexpression of the LbRos3 subunit in the *L. braziliensis* promastigote and intracellular amastigote stages restored miltefosine uptake and sensitivity to levels closer to those of *L. donovani*.

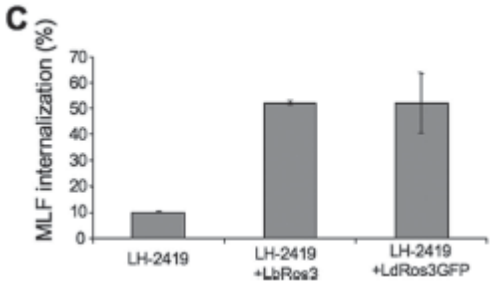
Figure 29: Overexpression of LbRos3 increases LbMT trafficking to the plasma membrane and miltefosine uptake and sensitivity in *L. braziliensis* promastigotes.



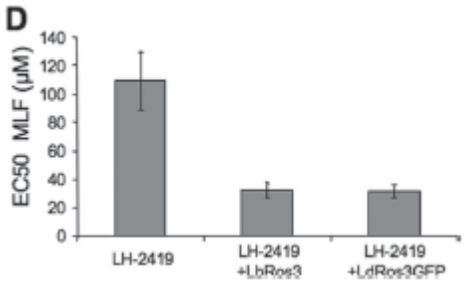
Overexpression of LbRos3 and LdRos3-GFP in crude membrane fractions of *L. braziliensis* LH-2419 promastigotes was detected by immunoblotting with anti-LdRos3 antibodies. The positions of molecular mass markers (kilodaltons) are indicated on the left.



L. braziliensis LH-2419 promastigotes transfected with the *LbRos3* or *LdRos3-GFP* construct expressed increased levels of LbMT and LbRos3 or LdRos3-GFP, at the plasma membrane. Western blot assays representative of at least three independent experiments are shown. The positions of molecular mass markers (kilodaltons) are indicated on the left.



^{14}C-miltefosine internalization after 60 minutes in *L. braziliensis* LH-2419 promastigotes overexpressing LbRos3 and LdRos3-GFP. Bars shown are the mean standard deviation of three independent experiments, and results are expressed as a percentage of the internalization by wild-type *L. donovani*.



Miltefosine sensitivity after 72 hours of culture in *L. braziliensis* LH-2419 parasites overexpressing LbRos3 and LdRos3-GFP. The EC50s shown are the mean ± standard deviation of three independent experiments.

Late-stage promastigotes of both the *L. braziliensis* LH-2419 parental strain and LbRos3-overexpressing lines were used to infect mouse peritoneal macrophages. Infected cultures were maintained with different miltefosine concentrations for 72 hours. Results showed that the ratio (3.3) of IC_{50}s of miltefosine for parental and *LbRos3*-transfected *L. braziliensis* was similar in both the promastigote and amastigote life cycle stages (Table 20); transfected parasites remained infective and maintained virulence, as determined by the percentage of infected macrophages and the average number of amastigotes per infected macrophage.

Table 20: Miltefosine sensitivity profiles of promastigotes and intracellular amastigotes of *L. braziliensis* lines[a]

Line	EC_{50} (μM)[b] (RI)[c]		% Infection[d]	No. of amastigotes/ cell[e]
	Promastigotes	Amastigotes		
LH-2419	109.0 ± 20.1 (3.3)	17.9 ± 5.4 (3.3)	50 ± 6	4.6 ± 1.4
LH-2419 + LbRos3	32.4 ± 5.1	5.4 ± 0.5	45 ± 16	3.6 ± 0.9

[a] *L. braziliensis* (L-H2419) parasites were grown for 72 h in the presence of increasing concentrations of MLF as described in Materials and Methods. Subsequently, promastigote and amastigote viability was determined by using an MTT-based assay and DAPI staining, respectively. Data are the means ± the standard deviations of three independent experiments.

[b] Results are expressed as the MLF concentration necessary to inhibit parasite growth by 50% (EC_{50}).

[c] The resistance index (RI) was calculated by dividing the EC_{50} for LH-2419 by that for parasites overexpressing LbRos3 (LH-2419 + LbRos3).

[d] Percent infection indicates the percentage of macrophages infected in the absence of drug at the endpoint of the assay for *Leishmania* amastigotes.

[e] The number of amastigotes per cell is the average number of intracellular amastigotes per infected macrophage in the absence of drug after 72 h of infection.

Studies with L. tropica

Increased efflux of miltefosine (and other endogenous phospholipid analogues) leading to a decrease in drug accumulation in the parasites has been implicated in miltefosine resistance and was shown to be mediated through the overexpression of the *Leishmania* P-glycoprotein-like transporter (*Leishmania* ABCB1 or LtrMDR1); this transporter belongs to ATP-binding cassette (ABC) superfamily of transporters that are usually involved in drug resistance in microorganisms. Reduced expression of P-glycoprotein in the multi-drug resistant (MDR) line of *L. tropica* correlated with a significant decrease in miltefosine resistance. ABC transporters are required for the active outward transport of PC, alkyl-glycerophosphocholine and phosphocholine derivatives.[35, 36]

Studies with L. infantum

Like the ABC transporters reported in *L. tropica* (summarized above), there are other ABC transporters (LiABCG6 and LiABCG4 half-transporters) identified in *L. infantum*.[37, 38] The

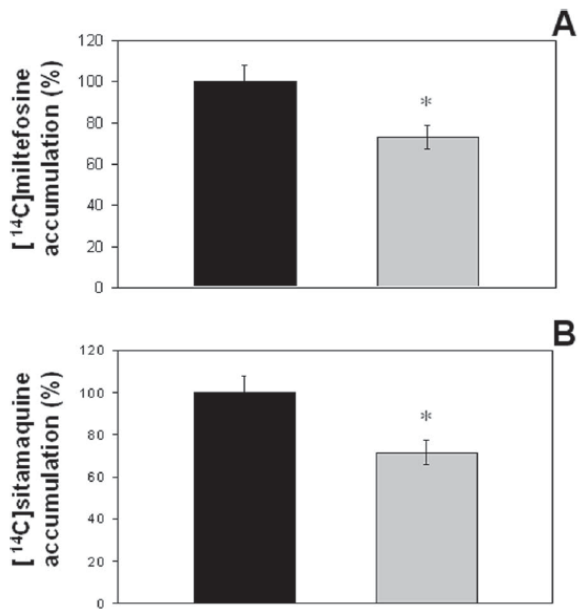
[35] Perez-Victoria JM, Perez-Victoria FJ, Parodi-Talice A, Jimenez IA, Ravelo AG, Castanys S, and Gamarro F. Alkyl-lysophospholipid resistance in multidrug-resistant *Leishmania tropica* and chemosensitization by a novel P-glycoprotein-like transporter modulator. Antimicrob Agents Chemother (2001) 45: 2468–2474.

[36] Perez-Victoria JM, Cortes-Selva F, Parodi-Talice A, Bavchvarov BI, Perez-Victoria FJ, Munoz-Martinez F, Maitrejean M, Costi MP, Barron D, Di Pietro Castanys AS, and Gamarro F. Combination of suboptimal doses of inhibitors targeting different domains of LtrMDR1 efficiently overcomes resistance of *Leishmania* spp. to miltefosine by inhibiting drug efflux. Antimicrob Agents Chemother (2006) 50: 3102–3110.

[37] Castanys-Munoz E, Alder-Baerens N, Pomorski T, Gamarro F, and Castanys S. A novel ATP-binding cassette transporter from *Leishmania* is involved in transport of phosphatidylcholine analogues and resistance to alkyl-phospholipids. Mol Microbiol (2007) 64: 1141–1153.

overexpression of these two *Leishmania*-specific ABC subfamily G-like transporters conferred resistance to not only miltefosine *in vitro*, but also to aminoquinolines (Figure 30).

Figure 30: Drug resistance profile for *L. infantum* promastigotes overexpressing LiABCG6a



Miltefosine and sitamaquine accumulation in *Leishmania* parasites. Mock-transfected (filled bars) and LiABCG6-overexpressing (shaded bars) promastigotes were incubated with ^{14}C-miltefosine (A) or ^{14}C-sitamaquine (B). After 30 min and 60 min, respectively, parasites were chilled on ice and washed with cold PBS, which, in the case of ^{14}C-miltefosine, contained 10 mg/mL BSA. Cell-associated radioactivity was quantified and normalized to the protein concentration. Data are expressed as the percentage of ^{14}C-miltefosine or ^{14}C-sitamaquine incorporated; the amount incorporated by the mock-transfected parasites is taken as 100%. Each bar represents the mean for four independent experiments performed in duplicate; error bars, standard deviations. Significant differences from results for mock-transfected parasites were determined by Student's test (*, $P < 0.01$ in panel A and P 0.005 in panel B).

Drug	IC_{50} (μM)[b] for parasites:				RI[c]
	Mock transfected	Overexpressing LiABCG6	Overexpressing LiABCG6-GFP	Overexpressing GFP-LiABCG6	
Miltefosine	6.0 ± 0.7	12.9 ± 1.5	5.6 ± 0.6	13.6 ± 1.2	2.2*
Edelfosine	4.8 ± 0.6	11.3 ± 0.2	4.8 ± 0.9	12.9 ± 0.5	2.4*
Perifosine	3.8 ± 0.6	11.0 ± 1.0	4.2 ± 0.2	12.2 ± 0.7	2.8*
Sitamaquine	7.4 ± 0.6	14.3 ± 0.2	7.8 ± 0.3	15.2 ± 0.4	1.9*
Chloroquine	6.4 ± 1.2	24.1 ± 1.2	N/D	N/D	3.8*
Mefloquine	5.2 ± 0.8	7.0 ± 0.9	N/D	N/D	1.4*
Primaquine	54.5 ± 0.6	54.9 ± 0.8	N/D	N/D	1
Ketoconazole	16.3 ± 1.3	19.3 ± 2.0	N/D	N/D	1.2
Camptothecin	17.1 ± 1.5	34.4 ± 1.8	N/D	N/D	2*

a Parasites were grown for 72 h at 28°C in the presence of increasing concentrations of drugs. Subsequently, cell viability was determined using an MTT-based assay. N/D, not determined.
b Data are means± standard deviations from six independent experiments.
c RI, resistance index, calculated by dividing the IC_{50} for LiABCG6-overexpressing parasites by that for mock-transfected parasites. Significant differences from results for parasites harboring the pXG control vector were determined by Student's test (*, P ,0.001).

Studies with L. major

Whole genome sequencing of *L. major* mutants revealed miltefosine resistance to be polyclonal with individual cells with varying susceptibilities and genotypes.[29] The markers of miltefosine resistance identified in this study and implicated in drug susceptibility were P-type ATPase and pyridoxal kinase and α-adaptin like protein. Pyridoxal kinase plays a vital role in the formation of pyridoxal-5′-phosphate (active vitamin B6).

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[38] Castanys-Munoz E, Perez-Victoria JM, Gamarro F, and Castanys S. Characterization of an ABCG-like transporter from the protozoan parasite *Leishmania* with a role in drug resistance and transbilayer lipid movement. Antimicrob Agents Chemother (2008) 52: 3573–3579.

3.4.1.2. *In vivo*

Relapse after miltefosine treatment of patients with visceral or cutaneous leishmaniasis has been reported.[39, 40, 41, 42, 43] Possible differences in immune response, drug pharmacokinetics, and exposure to antigens may affect clinical outcome. However, association of relapse (in patients with leishmaniasis or in animal models of infection) with phenotypic (a decrease in *in vitro* susceptibility of parasites to miltefosine) or genotypic changes has not been reported.

In a prospective cohort study in Nepal, visceral leishmaniasis patients treated with miltefosine were followed for up to 12 months after completion of therapy for clinical response as well as drug compliance, parasite drug resistance, and reinfection (Rijal *et al*., 2013).[43] All patients suspected of visceral leishmaniasis (fever of ≥2 weeks' duration with clinical splenomegaly) were admitted for a complete diagnostic workup; patients ≥2 years of age with parasitologically proven visceral leishmaniasis were enrolled. Diagnosis of visceral leishmaniasis was confirmed by demonstrating Leishman-Donovan (LD) bodies in a Giemsa-stained bone marrow aspirate or spleen aspirate, if the former was negative. Aspirates were inoculated and placed in culture for further identification. Patients were treated with miltefosine (2.5 mg/kg daily for 28 days). Clinical response was assessed at end of treatment, and at 3, 6 and 12 months after treatment using standard case definitions of treatment outcomes defined by the World Health Organization. Of the 217 patients enrolled, 120 were treated with miltefosine (Figure 31). The initial cure rate was 95.8%. In the following months, 24 patients who were initially cured relapsed and were confirmed parasitologically by bone marrow smears; relapse rates at 6 and 12 months were 10.8% and 20.0%, respectively. Relapse was most common among children (<12 years of age). None of the relapsing patients were HIV-positive.

There was no significant difference in miltefosine concentration at end of treatment between cured and relapsed patients; the mean (± SD) miltefosine whole blood concentrations were 46.7 μg/mL (± 15 μg/mL) and 44.5 μg/mL (± 16.6 μg/mL) for the cured and relapsed patients, respectively.

To differentiate relapse from reinfection, paired parasite isolates (pre-and post-relapse) from bone marrow aspirates of 8 patients were analyzed through kinetoplast DNA (kDNA) finger

[39] Pandey BD, Pandey K, Kaneko O, Yanagi T, and Hirayama K. Relapse of visceral leishmaniasis after miltefosine treatment in a Nepalese patient. Am J Trop Med Hyg (2009) 80: 580–582.

[40] Calvopina M, Gomez EA, Sindermann H, Coper PJ, and Hashiguchi Y. Relapse of New World diffuse cutaneous leishmaniasis caused by *Leishmania* (*Leishmania*) *mexicana* after miltefosine treatment. Am J Trop Med Hyg (2006) 2075: 1074–1077.

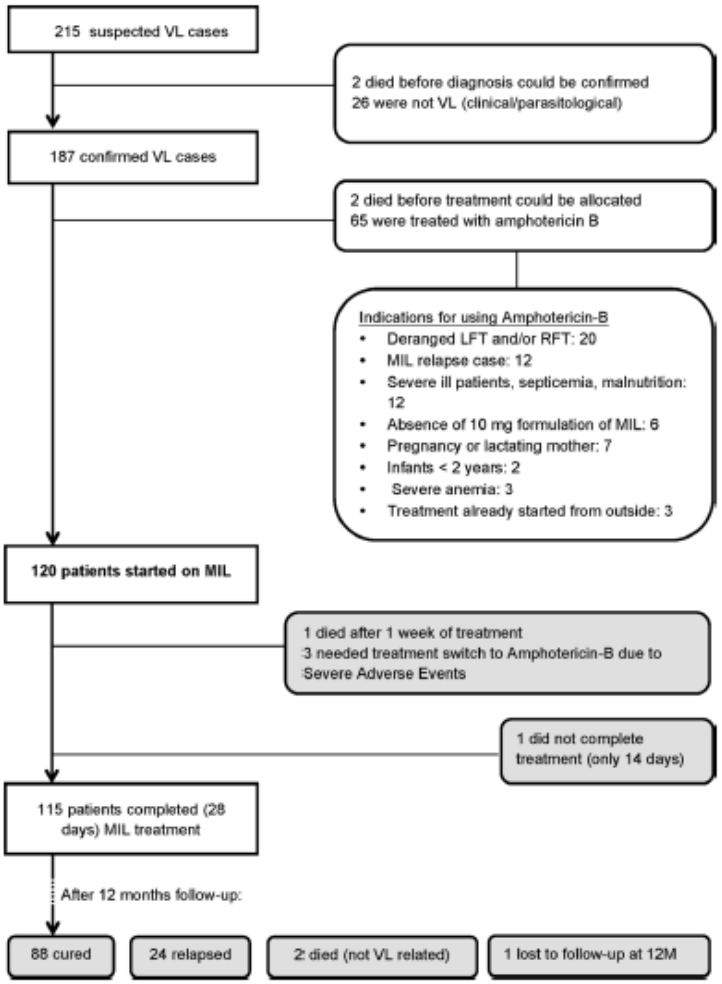
[41] Zerpa O, Ulrich M, Blanco B, Polegre M, Avila A, Matos N, Mendoza I, Pratlong F, Ravel C, and Convit J. Diffuse cutaneous leishmaniasis responds to miltefosine but then relapses. Br J Dermatol (2007) 156: 1328–1335.

[42] Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, Ostyn B, Boelaert M, Dujardin JC, and Chakravarty J. Efficacy of miltefosine in the treatment of visceral leishmaniasis after a decade of use in India. Clin Infect Dis (2012) 55 (4): 543-550.

[43] Rijal S, Ostyn B, Uranw S, Rai K, Bhattarai NR, Dorlo TPC, Beijnen JH, Vanaerschot V, Decuypere S, Dhakal SS, Das ML, Karki P, Singh R, Boelaert M, and Dujardin JC. Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. CID (2013) 56 (11): 1530-1538.

printing; a dendrogram was constructed using the unweighted pair group method with arithmetic mean to visualize the molecular similarity between the genotypes. The genotype of the parasites of pre- and post-treatment (relapse) isolates was identical suggesting that clinical relapses were not due to re-infection with a new strain. However, re-infection with the same strain/clone cannot be ruled out.

Figure 31: Recruitment, treatment, and follow-up of visceral leishmaniasis patients.

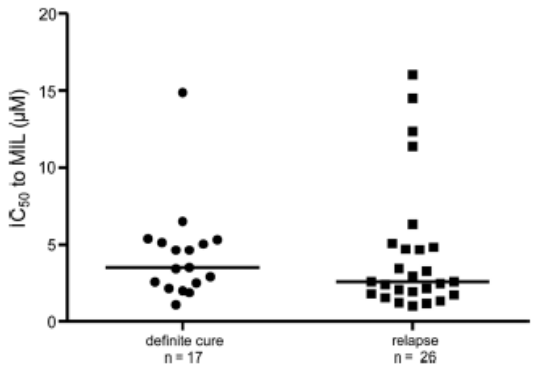


Abbreviations: LFT, liver function tests; MIL, miltefosine; RFT, renal function test; VL, visceral leishmaniasis.

In vitro susceptibility of promastigotes collected from patients before and at the end of treatment as well as from patients with definite cures (n=17) and relapses (n=26) was assessed by a promastigote assay.[23] The testing was performed at 2 sites (ITM and BPKIHS). Briefly, the promastigotes were cultured *in vitro* for about 12 passages; 10^5 promastigotes were incubated with different concentrations of miltefosine (0.64 µM 40 µM i.e., to 0.26 to 16.3 µg/mL) at 26°C. At the BPKIHS site, the activity of miltefosine was measured by adding trypan blue for vital staining followed by microscopy after 96 hours of incubation; at the ITM site, activity of promastigotes was measured by fluorescence method by addition of resazurin after 72 hours of incubation and cultures incubated for additional 24 hours. The results showed no difference in IC_{50} values of isolates from patients that were cured compared to relapsed patients (Figure 32). The authors state that there was no difference in *in vitro* susceptibility of pretreatment isolates (n

= 27) compared to post-treatment (end of therapy) isolates (n = 18) as well as the paired isolates, that is, a pre- and post-treatment isolate of the same patient (data not shown).

Figure 32: *In vitro* promastigote miltefosine susceptibility of isolates from definite cures and relapsed patients after treatment with miltefosine.



MIL, miltefosine.

The authors postulate that relapse could be due to the presence of reservoirs or sanctuary sites for the *Leishmania* parasite where the drug exposure may be lower or the parasite may exhibit different parasite dynamics compared to the systemic compartment.

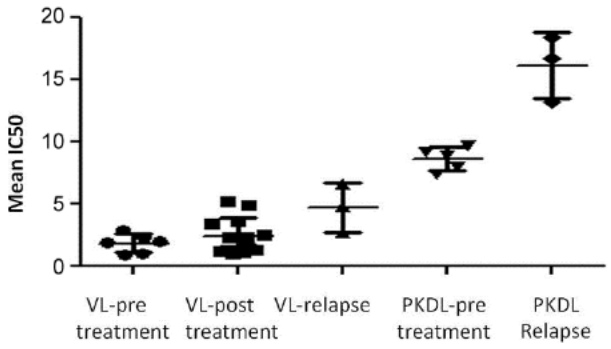
Another study (Bhandari *et al*., 2012),[44] reported *in vitro* susceptibility of clinical isolates of *L. donovani* collected from patients with visceral leishmaniasis or post kala-azar dermal leishmaniasis, before and after treatment with miltefosine, from a high endemicity area in Bihar, India. Miltefosine was administered for 28 days to visceral leishmaniasis patients (50 mg b.i.d.) and 60 days to patients with post kala-azar dermal leishmaniasis (50 mg, t.i.d.). Patients were followed for 1 year; patients that relapsed after initial cure were treated with amphotericin B. The *in vitro* susceptibility of the isolates was measured by the amastigote assay. Briefly, J774A.1 cells were infected with promastigotes at a ratio of 10:1. Cultures were incubated without or with different concentrations of miltefosine for 48 hours and number of amastigotes counted. Of the 30 isolates tested, 11 were pre-treatment isolates (6 from visceral leishmaniasis patients and 5 from post kala-azar dermal leishmaniasis patients), 13 post-treatment isolates from visceral leishmaniasis patients that were considered cured although remained parasite positive, and 6 from relapsed patients (3 visceral leishmaniasis and 3 post kala-azar dermal leishmaniasis patients (Table 21). The miltefosine IC_{50}s (0.95 to 2.91 µM i.e., 0.39 to 1.19 µg/mL; mean 1.86 µM i.e., 0.76 µg/mL) of the pretreatment isolates from patients with visceral leishmaniasis were lower than dose from patients with post kala-azar dermal leishmaniasis (7.37 to 9.69 µM i.e., 3.00 to 3.95 µg/mL; mean 8.63 µM i.e., 3.52 µg/mL). The miltefosine IC_{50}s for the isolates from 3 visceral leishmaniasis patients that relapsed were 2.66, 4.84, and 6.67 µM (i.e., 1.08, 1.97, 2.72 µg/mL, respectively; mean 4.72 µM i.e., 1.92 µg/mL) at month 7, 6, and 4, respectively; for the visceral leishmaniasis patients that were cured, although parasite positive, the miltefosine IC_{50}s ranged from 1.02 to 5.20 µM (i.e., 0.42 to 2.12 µg/mL; mean 2.43 µM i.e., 0.99 µg/mL) at the end of treatment. For the post kala-azar dermal leishmaniasis patients that relapsed after miltefosine treatment, the miltefosine IC_{50}s were 13.26, 16.70, and 18.45 µM i.e.,

[44] Bhandari V, Kulshrestha A, Deep DK, Stark O, Prajapati VK, Sundar S, Schonian G, Dujardin JC, and Salotra P. Drug susceptibility in *Leishmania* isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. PLoS Negl Trop Dis (2012) 6 (5): e1657.

5.40, 6.81, and 7.52 µg/mL (mean 16.14 µM i.e., 6.58 µg/mL), at month 12, 18, and 32, respectively. Miltefosine treatment does not appear to alter paromomycin IC_{50} values. No mutations were observed in the transport machinery (LdMT and LdRos3) genes.

Table 21: Drug susceptibility of *Leishmania donovani* clinical isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis.

doi:10.1371/journal.pntd.0001657.t001



In another study,[45] a gradual decrease of the miltefosine susceptibility of *L. infantum* isolates from a non-responsive HIV patient with visceral leishmaniasis was associated with the occurrence of a single nucleotide polymorphism in the LdMT gene, L832F, which reverted back to the wild-type allele 3 years after withdrawal from miltefosine. The IC_{50} values increased about 10-fold from 5 µM to 50.1 µM i.e., 2.0 to 20.4 µg/mL (Table 22); the IC_{50} value of the isolate 6 months after withdrawal of miltefosine therapy was 6-fold higher than the pre-treatment isolate. Overall, the study suggests that point mutations within the LdMT locus may be responsible for the resistant phenotype through inactivation of the protein.

Table 22: Comparisons of IC_{50} for amphotericin B and miltefosine against promastigotes and axenic amastigotes and distribution of *LdMT* SNPs in *Leishmania infantum* isolates and reference strains*

				IC_{50}, µmol/L ± SEM				
				AmpB		Miltefosine		
Isolate	Year	AmpB	Miltefosine	Promastigotes	Axenic amastigotes	Promastigotes	Axenic amastigotes	*Ldmt* SNP
–	1998	3 mg/kg/d × 5 d; then 1×/wk × 5 wk	–	–	–	–	–	–
S_1	2000	3 mg/kg/d × 5 d; then 1×/wk × 5 wk	–	0.09 ± 0.04†	0.10 ± 0.03	7.14 ± 0.56†	5.00± ± 0.7†	L832
–	2001	4 mg/kg/d × 5 d; then 1×/wk × 5 wk	50 mg 2×/d	–	–	–	–	–
S_3	2005	4 mg/kg/d × 5 d;		0.13 ± 0.03	0.20 ± 0.03	25.93 ± 1.46†	21.00 ± 1.50†	832L/F
S_4		then 1×/wk × 5 wk‡		0.24 ± 0.01†	0.15 ± 0.02	27.89 ± 1.76†	31.90 ± 1.60†	
–	2007	4 mg/kg/d × 5 d; then 1×/wk × 5 wk		–	–	–	–	–
S_6	2008	4 mg/kg/d × 5 d; then 1×/wk × 5 wk	–	0.16 ± 0.03	0.11 ± 0.03	44.30 ± 3.70†	50.10 ± 1.00†	832F
S_7	2010	4 mg/kg/d × 5 d; then 1×/wk × 5 wk	–	–	–	–	–	L832
Reference strain								
LV9 WT				0.03 ± 0.02	0.02 ± 0.05	4.46 ± 0.29†	6.20 ± 0.3	L832
LV9 Miltefosine-R				0.22 ± 0.04	0.70 ± 0.09	45.84 ± 2.40†	54.20 ± 2.20†	832F
DD8 WT				0.06 ± 0.02†	0.05 ± 0.03†	17.40 ± 1.70	12.40 ± 1.50	L832
DD8 AmpB-				1.42 ± 0.06†	1.00 ± 0.07†	15.20 ± 1.00	10.30 ± 1.20	L832

*IC_{50}, 50% inhibitory concentration; AmpB, amphotericin B; *Ldmt*, *Leishmania donovani* miltefosine transporter gene; SNP, single-nucleotide polymorphism; –, assay not performed because sample unavailable or not cultivable; WT, wild type; R, resistant.
†Significance was analyzed by using the nonparametric Mann-Whitney U test to compare the IC_{50} of the isolates with the IC_{50} of reference strains; $p<0.01$ was considered significant. IC_{50} of AmpB and miltefosine was compared with IC_{50} of reference strains and S_1/S_3, S_1/S_4, and S_1/S_6. Miltefosine: S_1/S_3, S_1/S_4, S_1/S_6, S_4/S_6; $p<0.01$. AmpB: S_1/S_4 significant $p<0.01$; S_1/S_3, S_1/S_6 not significant.
‡For each relapse.

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[45] Cojean S, Houze´S, Haouchine D, Huteau F, Lariven S, Hubert V, Michard F, Bories C, Pratlong F, Le Bras J, Loiseau PM, and Matheron S. *Leishmania* resistance to miltefosine associated with genetic marker. Emerging Infect Dis (2012) 18: 704–706.

Comments:
Overall, the in vitro and clinical studies show a potential for development of resistance to miltefosine. Drug resistance could be due to a reduction in drug concentration within the cell. Such a defect was shown to be associated with

- *inactivation or low expression of the miltefosine transport complex in L. donovani and L. braziliensis. This transporter complex is important for maintaining the integrity of the cell membrane. Some strains of L. braziliensis are considered to be intrinsically resistant to miltefosine due to a low expression of miltefosine transporter and protein complex.*
- *overexpression of ABC transporters located in the plasma membrane of L. infantum and L. tropica.*
- *changes in the length and level of unsaturation of fatty acids, as well as a reduction in ergosterol levels suggesting that fatty-acid and sterol metabolism are probably targets for miltefosine resistance.*
- *point mutations (e.g., L832F) within the LdMT locus; in one patient with visceral leishmaniasis, point mutations were observed that may be responsible for the resistant phenotype through inactivation of the protein.*

3.4.2. Cross Resistance

An *in vitro* study suggests cross-resistance between miltefosine and another alkyl-phospholipid (edelfosine). No studies supporting cross-resistance between miltefosine and drugs currently available in the world for the treatment of leishmaniasis were available for review.

4. OVERVIEW OF CLINICAL PHARMACOLOGY

The applicant states miltefosine is (b) (4)

(b) (4)

(b) (4)

(b) (4)

5. CLINICAL MICROBIOLOGY

The applicant has submitted clinical studies to support the efficacy and safety of miltefosine for the treatment of visceral, mucosal, and cutaneous leishmaniasis. The applicant also included one study in patients with diffuse cutaneous leishmaniasis; although this indication is not being pursued by the applicant, the study was reviewed to evaluate the activity against *Leishmania* species (*L. amazonensis*) prevalent in Venezuela. Miltefosine (IMAPVIDO) was administered orally in all clinical trials unless specified otherwise.

5.1. Visceral leishmaniasis

The efficacy and safety of miltefosine was measured in patients with visceral leishmaniasis in one pivotal study (Study 3154) in India, a supportive study (Study Z025) in Ethiopia. Additional study reports available for review include dose ranging studies in India, phase 4 studies in the Indian subcontinent conducted after registration of the drug in India, and compassionate use in Europe (Spain, France, Italy, and Portugal); however, datasets were not available.

5.1.1. Study 3154 and 3154a

This was a phase 3, randomized, open label, active controlled, multicenter trial to evaluate the efficacy (initial and final cure) and tolerability of oral miltefosine (2.5 mg/kg/day for 28 days) compared to amphotericin B administered by intravenous infusion (15 mg/kg every other day for 30 days), a standard therapy for the treatment of visceral leishmaniasis in India (Study report D-18506/2200000052, dated November 28, 2001; Sundar *et al*., 2002[46]). Based on epidemiological findings, *L. donovani* is known to be the prevalent *Leishmania* species in this area. The **primary objective** was assessment of final cure rates i.e., documented eradication of parasites (initial cure) and disappearance of clinical signs and symptoms attributable to leishmaniasis for at least six months.

Secondary objectives were

- assessment of the initial cure,
- assessment of clinical response at end of study treatment, and
- characterization of the safety of the proposed miltefosine schedule.

Study design

Inclusion criteria

- Adolescents (12 -17 years of age) and adults.
- Newly diagnosed or resistant/relapsing visceral leishmaniasis, confirmed by splenic or bone marrow aspiration.
- Clinical signs and symptoms compatible with visceral leishmaniasis (e.g., fever, splenomegaly, anemia).

[46] Sundar S, Jha TK, Thakur CP, Engel J, Sindermann H, Fischer C, Junge K, Bryceson A, and Berman J. Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med (2002) 347:1739-1746.

Exclusion criteria

Lack of suitability for the trial

- Concomitant treatment with other anti-leishmaniasis drugs.
- Failure of prior amphotericin B therapy (i.e. lack of initial cure at adequate dose and regimen, or lack of tolerability); this criterion was added by amendment no. 2 to the clinical protocol.
- Any condition which compromised ability to comply with the study procedures.

Safety concerns

- Thrombocyte count $<50 \times 10^9$/L.
- Leukocyte count $<1 \times 10^9$/L.
- Hemoglobin < 6.0 g/100 mL.
- ASAT, ALAT, AP ≥3 times upper limit of normal range.
- Bilirubin ≥2 times upper limit of normal range.
- Prothrombin time ≥5 seconds above control.
- Serum creatinine or BUN ≥1.5 times upper limit of normal range.
- Major surgery within last 2 weeks.
- Any non-compensated or uncontrolled condition, such as active tuberculosis, malignant disease, severe malaria, HIV, or other major infectious diseases.
- Lactation, pregnancy (to be determined by adequate test) or inadequate contraception in females of childbearing potential for treatment period plus 2 months.

The study was performed in 3 center in Muzaffarpur (Centers 1 and 2), and Patna (Center 3) India. Clinical and parasitological responses were assessed at baseline and at different follow-up visits up to 12 months at all 3 centers (Table 24). At one of the center (Center 2), additional follow-up was performed at 12 months post-treatment. Clinical response was based on signs/symptoms attributable to visceral leishmaniasis that includes spleen size, WBC, hemoglobin and thrombocytes, and fever [kala-azar (to be differentiated by investigator from fever for other reasons)]. In patients with an aspirate score = 1 at end of treatment another aspirate was evaluated 4 weeks after the end of treatment. In patients with clinical signs/symptoms attributable to visceral leishmaniasis during follow up, an aspirate was evaluated to verify the response status.

According to amendment no. 2 of the clinical protocol, for final assessment all smears were read by the same pathologist (b) (4) for all three study centers (unblinded). One out of ten slides marked with a code number only was forwarded to (b) (4) for review/external quality control under blinded conditions. Parasite density was graded microscopically (10X eyepiece and 100X objective) from 0 (no amastigotes per 1000 fields) to 6+ (> 100 amastigotes per field).

Table 24: Study 3154 - Time schedule

FLOW CHART									
	Visit	1	2	3	4	5	6[f]	7	8[h]
Assessment	Day	Before[a]	Day 1	Day 7	Day 14	Day 21	Day 28/ 30* or end of treatm.	4 wks after end of treatm.	6 month after end of treatm.
Informed consent		X							
Randomization		X							
Medical History		X							
History of leishman.		X							
Chest x-ray		X							
ECG		X		X	X	X	X		
Parasitology (stool / blood)		X							
Pregnancy test (females of child-bearing potential)[c]		X					X		
HIV test		X							
Efficacy - spleen or bone marrow aspirate[b]		X					X	(X)[d]	(X)[g]
- splenomegaly		X		X	X	X	X		X
- temperature		X	daily						X
Laboratory[e] - Hemogram		X		X	X	X	X		X
- Chemistry		X		X	X	X	X		X
- Urinalysis		X					X		
Vital parameters		X	X	X	X	X	X		X
Ophthalmologic exam. *(according to amendment no. 1)*		X					X		X
Medication			daily (miltefosine) every-other-day (amphotericin B)						
Global assess. Tolerability							X		
Concomit. diseases		X	monitoring of concomitant diseases						
Concomit. treatments		X	documentation of any changes						
Adverse events		X	documentation as occurring[g]						

* day 28 for miltefosine / day 30 for amphotericin B

a Within 7 days before 1st dose

b If spleen was too small or if bone marrow aspiration was done at pretreatment, bone marrow aspirates should be performed to determine presence of parasites.

c Screening assessment performed in the first half of menstrual cycle (≤ day 10 of cycle): in case of negative pregnancy test prior to randomization, treatment could be started without waiting period. Screening assessment performed in the second half of menstrual cycle (> day 10): pregnancy test to be followed by a two weeks waiting period during which adequate contraceptive measures were to be used. After waiting period a confirmative pregnancy test was to be done prior to randomization.

d In case of aspirate score = 1 at end of treatment a re-aspirate 4 weeks after end of treatment was to be done

e Additional examinations to be performed as medically indicated. Addition by amendment no. 3:. In case of CTC grade 3 elevation of liver enzymes, control exams on the next day (for confirmation) and then every 3 days are to be performed until the value(s) for liver enzyme(s) were ≤ CTC grade 2.

f A full assessment should be done after treatment discontinuation. The reason(s) for discontinuation were to be documented. This also applied to patients withdrawing from the study for whatever reason.

g For follow up of adverse events persisting at the end of treatment ➔ chapter 8.4 of protocol.

h The final evaluation was scheduled 6 months after end of treatment. In a patient with a relapse, the final evaluation was to be performed when the relapse was diagnosed. Only if a relapse was assumed due to clinical signs / symptoms attributable to visceral leishmaniasis, a splenic / bone marrow aspirate was to be performed at final evaluation.

Response criteria were graded as follows:

Final cure

Final cure was defined as initial cure followed by 6 months follow up without relapse and absence of clinical signs or symptoms attributable to visceral leishmaniasis (Figure 32).

Initial cure

Initial cure was defined as eradication of parasites at the end of treatment or within 4 weeks thereafter (Figure 32).

Treatment failure

- Spleen/bone marrow aspirate score > 1 at end of treatment (no adequate response to therapy).
- Spleen/bone marrow aspirate score > 0 at re-assessment 4 weeks after end of treatment.
- Spleen/bone marrow aspirate score > 0 any time after 4 weeks after end of treatment (relapse).

Treatment failure at any time after end of study treatment: Patients were treated with rescue medication (AmBisome®) provided by the applicant.

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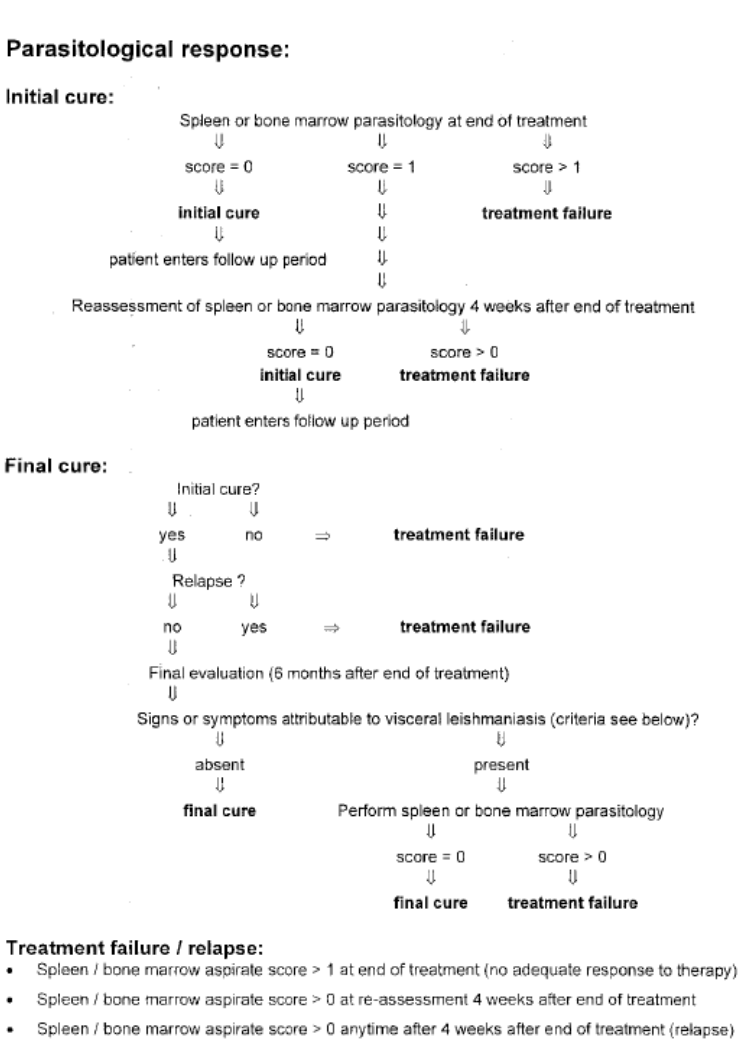
Figure 32: Study 3154 - Criteria for evaluation of response



Results

Overall, the disease status at baseline was similar in the two groups; in the miltefosine group 214 (71.6%) patients had newly diagnosed, non-pretreated leishmaniasis. The remaining patients had received prior therapy for their disease: 69 patients (23.1%) were primary unresponsive, 16 patients (5.4%) had experienced a relapse. In the amphotericin B group 71 patients (71.7%) were newly diagnosed cases, 21 patients (21.2%) were primary unresponsive, 7 patients (7.1%) had a relapse. Of the 398 patients (299 in the miltefosine arm and 99 in the amphotericin B arm) exposed to at least one dose of study medication [intention to treat (ITT) population], 17 patients were excluded from the per protocol (PP) population (287 in the miltefosine arm and 94 in the amphotericin B arm), because of premature discontinuation of treatment or follow-up due to reasons other than treatment failure.

The results showed miltefosine to be as effective as amphotericin B for the treatment of visceral leishmaniasis at the end of therapy and 6 month follow-up in both ITT and PP population (Table 25). There was no apparent difference in the cure rate depending on the history of the disease in relation to prior therapy or parasitology score.

Table 25: Study 3154 – Summary of clinical and parasitological responses

Treatment group/ species	End of therapy n/N (%)			Follow up at 6 months n/N (%)		
All species were *L. donovani* by epidemiology	Clinical success (Initial cure)[a]	Proven parasitological eradication n negative/ n tested	Presumed parasitological eradication	Clinical success (Final cure)	Proven parasitological eradication n negative/ n tested	Presumed parasitological eradication n cured/ n not tested
ITT						
Miltefosine	294/299 (98%)	294/299 (98%)	NA[b]	282/299 (94%)	19/28 (67.9%)	263/271 (97.0%)
Amphotericin B	96/99 (98%)	97/99 (98%)	NA	96/99 (97%)	1/1 (100%)	95/98 (97%)
Total	391/398 (98%)	391/398 (98%)	NA	378/398 (95%)	20/29 (69.0%)	358/379 (94.4%)
PP						
Miltefosine	287/287 (100%)	287/287 (100%)	NA	279/287 (97%)	19/27 (70.4%)	260/260 (100.0%)
Amphotericin B	94/94 (100%)	94/94 (100%)	NA	94/94 (100%)	1/1 (100%)	93/93 (100%)
Total	381/381 (100%)	381/381 (100%)	NA	373/381 (98%)	20/28 (71.4%)	353/353 (100.0%)

[a] Initial cure was defined as clinical cure + parasitological cure at end of treatment or if parasitological score was 1 at end of treatment, then score was negative on retest

[b] Not applicable as parasitology was performed on all patients

"Proven parasitological eradication" was the number of patients for whom parasitological follow-up was performed and shown to be parasitologically-negative. At the end of therapy, "Presumed parasitological eradication" was the difference between "initial cure" minus the number tested for parasites.
For a subject to be considered as clinical cured, they must also have had parasitological cure at the end of therapy, or if the parasitology score was equal to 1 at the end of therapy, a repeat test two weeks later had to be negative, at which point the patient was considered to have "initial cure".

The Medical Officer considered 12 patients in the miltefosine arm and 2 patients in the amphotericin B arm as failures; therefore, the number of patients listed in the box are different from those in the clinical review. However, it does not change the conclusion.

Initial cure

In the miltefosine group 294 of 299 patients (98.0%) were initially cured; five patients (1.7%) were not assessable. In the amphotericin B group 98 of 99 (99.0%) patients had an initial cure and one patient (1.0%) was not assessable. No amastigotes were reported in a majority of the subjects in either of the treatment arms.

Final cure rates

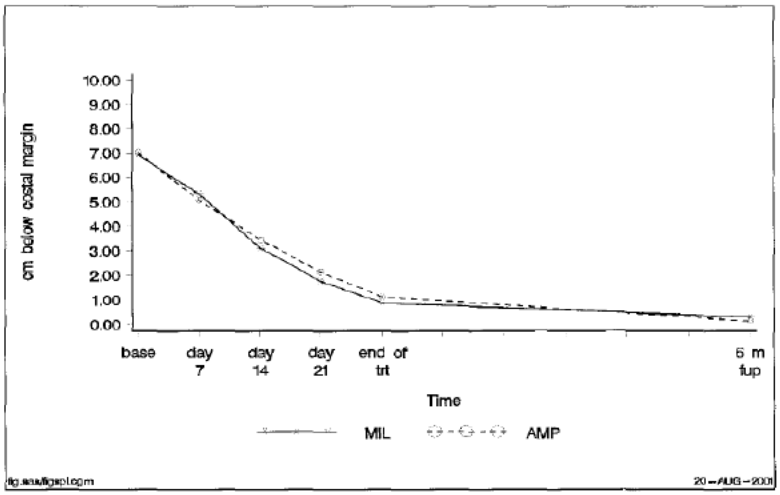
Cure rates at 6 months were 94% in the miltefosine arm and 97% in the amphotericin B arm. There were 9 patients that relapsed (aspirate positive) in the miltefosine arm. No relapses were reported in the amphotericin B arm.

Spleen size

Spleen size, assessed by palpation and size [documented as length (cm) of longest axis], decreased rapidly in both treatment groups (Figure 33). At the 6-month follow-up, spleen was no

longer palpable in 262 of 291 miltefosine treated patients (90.0%) and in 88 of 96 amphotericin B treated patients (91.7%).

Figure 33: Study 3154 - Spleen size (mean)



MIL: N = 299→291; AMP: N = 99→96

Body temperature

After about one week, fever had completely resolved in miltefosine-treated patients and body temperature remained low until end of treatment. In contrast, each infusion of amphotericin B was followed by an increase in body temperature of about 2°C.

Long term follow-up

Follow-up at 12 months after end of treatment was performed at one of the centers (Center 2). Of the 143 patients, 108 were treated with miltefosine, and 35 patients with amphotericin B. One patient in the amphotericin B was lost to follow-up. At the 12 month follow-up visit, 111 of the 143 patients were without any signs or symptoms typical for leishmaniasis and classified as still cured. In 32 patients (23 miltefosine, 9 amphotericin B) symptoms potentially indicative of leishmaniasis were reported. Only one of 108 patients who had been treated with miltefosine had a recurrence of the disease after the prolonged follow-up; an aspirate was analyzed and was positive for parasitological findings.

Comments:

- *Similar final cure rates were achieved after oral administration of miltefosine (100 mg/kg/day for 28 days) and intravenous administration of amphotericin B (1 mg/kg every other day for 30 days) in patients with visceral leishmaniasis in India. Miltefosine was equally effective in patients pre-treated and non-pretreated with pentavalent antimonial drugs. Parasitological confirmation at the end of treatment and follow-up visits was performed if clinically indicated.*
- *Parasitological diagnosis at baseline was based on smear/aspirate by microscopy. No efforts were made to identify Leishmania species. However, based on epidemiologic findings, L. donovani is known to be the etiological agent for visceral leishmaniasis in India.*
- *All the documented relapses occurred in the miltefosine arm, suggesting a potential for development of resistance.*

5.1.2. Study Z025

This was a randomized, open label trial to determine the efficacy and tolerability of miltefosine (2.5 mg/kg/day for 28 days) compared with that of intramuscular sodium stibogluconate (20 mg/kg/day for 30 days), in male patients with visceral leishmaniasis in Kafta Humera Woreda, Ethiopia (Study report dated June 13, 2012; Ritmeijer *et al*., 2006[47]). Based on epidemiological findings, *L. donovani* is known to be the prevalent *Leishmania* species in this area. The applicant states that patients in this area are supposedly "sicker" compared to those in India.

Primary endpoints

- Cure rate at 6 months of follow-up after the last dose; the final cure was based on PP population i.e., the population that was randomized and was available for evaluation at 6 months.
- Death rate by the end of therapy; death rate was generated *post hoc* when the high death rate in this study was recognized.[48]

Secondary endpoints

- Initial cure rate in the ITT population: initial cure was defined as clinically cured (i.e., alive and with fever clearance, diminution of spleen size, increased hemoglobin, or weight gain at the end of therapy, and without parasites at the end of therapy if parasite aspiration was performed.

Study design

Inclusion criteria

- Fever for 2 weeks or more (with exclusion of malaria).
- Either splenomegaly or wasting.
- A positive *Leishmania* direct agglutination test (DAT) (Meredith *et al.,* 1995[49]) defined as a titer of >1/6400, or if an intermediate titer of 1/800 to 1/3200 was found, an organ aspirate demonstrating parasites on smear (Ritmeijer *et al.,* 2006[47]).

  The applicant states that the World Health Organization (WHO) case definition of visceral leishmaniasis was used for initial screening: a history of fever for 2 weeks (with malaria excluded) in combination with wasting, and either splenomegaly or lymphadenopathy (WHO-1996, MSF-2004). For patients whose illness met this case definition, visceral leishmaniasis was confirmed by a high titer *Leishmania* DAT titer ≥ 1:6400 (Meredith *et al*., 1995[49]). In patients with an intermediary DAT titer (1:800–1:3200), splenic or lymph node

[47] Ritmeijer K, Dejenie A, Assefa Y, Hundie TB, Mesure J, Boots G, den Boer B, and Davidson RN. A comparison of miltefosine and sodium stibogluconate for treatment of visceral leishmaniasis in an Ethiopian population with high prevalence of HIV infection. Clin Infect Dis (2006) 43: 357-64.

[48] The applicant states that patients with clinical/parasitological failure will eventually die if not given rescue therapy and "clinical/parasitological failure" is in reality a surrogate marker for "death". For a trial in which death occurred with sufficient frequency to be statistically analyzable, having the primary outcome variable measured the true visceral leishmaniasis endpoint rather than a surrogate endpoint and had inherent value. "Death rate by the end of therapy" rather than "Death rate by the end of the study" was used as the mortal endpoint, since death during treatment was observed, whereas death between the end of treatment and the 6 month follow-up was not observed and cannot be attributed to disease.

[49] Meredith SE, Kroon NC, Sondorp E, Seaman J, Goris MG, van Ingen CW, Oosting H, Schoone GJ, Terpstra WJ, and Oskam L. Leish-KIT, a stable direct agglutination test based on freeze dried antigen for serodiagnosis of visceral leishmaniasis. J Clin Microbiol (1995) 33:1742-1745.

aspiration was performed, and visceral leishmaniasis was confirmed by microscopic examination. Persons with suspected visceral leishmaniasis with a negative DAT titer (≤ 1:400) were evaluated for alternative illnesses and were retested if signs and symptoms persisted. Severely ill patients were aspirated without delay, so that a diagnosis could be made as quickly as possible. Patients with previous anti-leishmanial treatment were only admitted if they had a positive aspirate result.

- Male subjects ≥ 15 years of age.

Exclusion criteria

- Patients were excluded if they were thought unlikely to survive 1 month's treatment because of severe co-morbidity.

There was no prohibition with respect to prior or concomitant therapy. Patients with prior treatment for leishmaniasis were enrolled into the study. It appears that concomitant treatment for leishmaniasis did not occur as the applicant states that concomitant treatment for leishmaniasis was impossible since all medical therapies were administered by the clinical trial staff.

The patients were assessed for clinical and parasitological response at different time points (Table 26). The quality control of the DAT test for visceral leishmaniasis diagnosis and HIV tests was as per the standards at the hospital.

The applicant did not provide a definition of relapse; however according to the publication (Ritmeijer *et al.*, 2006[47]) relapse was defined as clinical symptoms of infection with parasitological confirmation within the 6-months follow-up period. If relapse was suspected (according to clinical case definition) an aspirate was performed to confirm visceral leishmaniasis. If no relapse occurred by 6 months after discharge, the patient was considered to be finally cured.

In patients with a palpable spleen at the end of treatment, a splenic aspirate was performed; in other patients, a lymph node aspirate was performed. The splenic or lymph node aspirate was subjected to microscopic analysis for *Leishmania* parasites. The microscopist was blinded to the treatment group. The slides were numbered and kept for verification by expert microscopist in a reference laboratory and the verifying microscopist was also unaware of the treatment group.

In patients without palpable spleens or lymph nodes, cure was established clinically.

Table 26: Study Z025 - Study assessments schedule

Assessment	Screening Period	Treatment Period	End treatment	End 6 month Follow up
Demographics	X			
Medical history	X		X	X
Physical exam	X		X	X
Vital signs	X	fever daily		
Walking status	X			
Weight	X	weekly	X	X
Spleen size	X		X	X
Parasitology/serology	X		parasitology	
Laboratory				
HIV		X		
Hematocrit	X		X	
Drug Treatment				
Miltefosine		Days 1-28		
SSG		Days 1-30		
Adverse events		daily		
Concomitant medications		daily		

Results

Of the 580 randomized subjects, 290 were in the miltefosine arm and 290 in the sodium stibogluconate arm. None of the baseline characteristics found on physical examination in the patients who died differed between the treatment groups. Baseline laboratory values that are biomarkers for disease severity such as hematocrit (reflecting pan-cytopenia), DAT score, and splenic aspirate score were similar between the 2 treatment groups (Table 27). Parasite density was higher in HIV^+ patients compared to HIV^- subjects.

The initial cure rates in the ITT population (secondary endpoint) and the final cure rates in the PP population (secondary endpoint) were similar in the miltefosine and sodium stibogluconate arm; however, the number of deaths was lower in the miltefosine arm compared to those in the comparator arm (Figure 34). Relapse rates were higher in the miltefosine group compared to the comparator group (Figure 34).

Although the protocol specified a separate analysis of cure rates by HIV status, the HIV status for individual subjects was not available to the applicant; the publication by Ritmeijer *et al.* (2006),[47] reported the findings by HIV serostatus. The initial and final cure rates were lower in HIV^+ patients compared to HIV^- subjects. Treatment with miltefosine was as effective as sodium stibogluconate in non–HIV-infected patients with visceral leishmaniasis, however, efficacy was lower in the miltefosine arm than sodium stibogluconate among HIV co-infected patients. Miltefosine was safer than sodium stibogluconate in both HIV^+ and HIV^- groups.

Relapse rates were higher in the HIV^+ patients compared to HIV^- subjects suggesting lower cure rates in immunocompromised subjects (Table 27 and Figure 34).

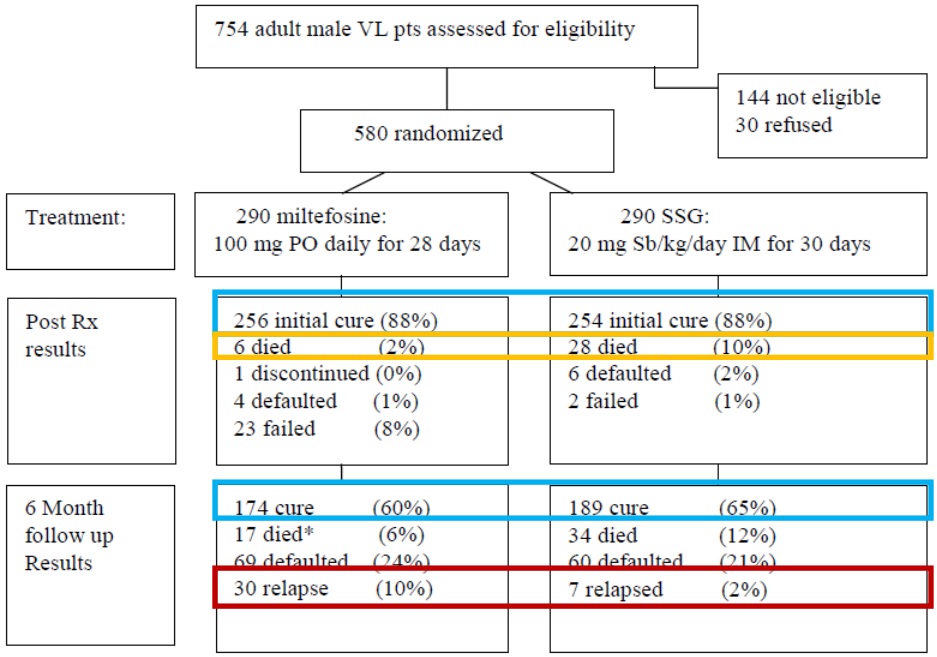
Table 27: Study Z025 - Baseline characteristics of patients with and without HIV co-infection (by HIV serostatus).

Baseline characteristics

Events during treatment and outcomes of patients with and without HIV co-infection randomized to receive miltefosine or sodium stibogluconate

Source: Ritmeijer *et al*., 2006[47]

Figure 34: Study Z025 - Disposition of subjects and cure rates



SSG: sodium stibogluconate
* Note that publication states that 17 patients died, but CRFs for death available to the applicant consisted of 6 subjects who died during therapy plus 9 who died between therapy and the 6-month follow up.

The authors state that final outcomes among patients who had received previous treatment for visceral leishmaniasis were significantly worse, with higher relapse rates [10 (29%) of 34] and death rates [10 (29%) of 34], compared to those previously untreated patients, [41 (7.5%) of 546 died, and 27 (4.9%) of 546, respectively].

Of the 30 patients who experienced relapse in the miltefosine group, 24 were cured after re-treatment with a full course of sodium stibogluconate; in the sodium stibogluconate group, 3 of the 7 patients who experienced relapse were cure after re-treatment.

Comments:

- *Final cure rates, at the end of treatment or at follow-up, were similar in patients treated with miltefosine or sodium stibogluconate. The mortality rate was lower in the miltefosine group, compared to the comparator (sodium stibogluconate) group. However, relapse rates were higher in the miltefosine group compared to the comparator.*
- *The cure rates were lower and relapse rates higher in HIV^{+} subjects compared to HIV subjects.*
- *The cure rates were lower in this study in Ethiopian patients compared to Study 3168 in Indian patients; this could be due to differences in severity of the disease, host factors, or differences in Leishmania strain.*
- *Diagnosis at baseline was based on clinical features (splenomegaly), DAT and parasitological observations by microscopy of organ aspirate.*

  The DAT used for initial diagnosis is not FDA cleared. The authors have referred to a study by Meredith et al. (19949) performed in Amsterdam.[49] Briefly, DAT was performed with a

freeze-dried (FD) antigen of L. donovani using a kit and anti-Leishmania antibodies were detected; the samples tested included serum samples from

- *50 patients of which 21 were from patients from Perkerra region in Kenya and 29 from patients who presented at the kala-azar treatment center in Nimne in Southern Sudan. Twenty serum samples from this last group plus four serum samples from other visceral leishmaniasis patients from Nimne were available for comparison of titers obtained with FD and aqueous suspension antigens in the field in southern Sudan.*
- *52 healthy controls living in West Africa.*
- *29 controls living in areas of Sudan in which visceral leishmaniasis is endemic; they were healthy individuals who had no history of visceral leishmaniasis and no clinical symptoms of the disease.*
- *354 patients with a variety of diseases other than visceral leishmaniasis, namely, 71 with toxoplasmosis (Europe), 30 with malaria (Burkina Faso), 18 with sarcoidosis (Europe), 21 with schistosomiasis (Mali), 30 with African Trypanosomiasis (Equatorial Guinea), 19 with Chagas disease (Brazil), 20 with tuberculosis (Europe), 30 with toxocariasis (Europe), 23 with amoebiasis (Europe), 7 with giardiasis (Europe), 20 with Crohn's disease (Europe), 45 with onchocerciasis (Ivory Coast), 10 with leprosy (Philippines), and 10 with autoimmune diseases (vasculitis, purpura vasculitis, psoriasis, pemphigus, systemic lupus erythematosus, rheumatoid arthritis, and cutaneous discoid lupus erythematosus; Europe).*

In general the FD antigen showed antibody titers that were one-dilution lower than the aqueous suspension. No cross-reactivity was reported against several pathogens tested. The sensitivity was 92% and specificity 99.7%. The authors concluded that the DAT using heat-stable FD antigen was a highly specific and adequately sensitive addition to the diagnostic armament for visceral leishmaniasis but that the main gain lies in its reproducibility combined with a long shelf life even under harsh conditions.

Please note that the DAT is not capable of distinguishing between past kala-azar, subclinical infection, and current disease. It is reported that increased levels of anti-Leishmania antibodies may be present for a long time after completion of treatment. On the other hand, if visceral leishmaniasis is associated with HIV infection or immunocompromised status other than HIV, a considerable number of patients lack detectable levels of antibodies. The authors recommend that, as with all serodiagnostic tests, the significance of positive or negative results in the DAT should always be judged against clinical data and the results of other diagnostic methods.

Overall, the use of the DAT test (in conjunction with clinical features and parasitological observations) for diagnosis as used in the Study Z-025 was appropriate.

No efforts were made to identify Leishmania species. However, based on epidemiologic findings, Ethiopia is known to be endemic for L. donovani.

5.1.3. Other studies

The applicant has provided a summary of additional studies that include dose ranging studies in adults and children. A majority of the studies were conducted in Indian subcontinent known to be endemic for *L. donovani* and are summarized in Table 28. The parasitological measurements in all the studies include examination of aspirates by smear, pre- and post-treatment, unless specified otherwise.

Table 28: Summary of visceral leishmaniasis clinical studies in different geographic regions

Study Number Geographic area (*Leishmania* spp)*	**Study Design and Results**
033 (Pilot) Varanasi, India (*L. donovani*)	Open label, non-controlled, sequential group, dose ranging (50 mg every other day and 100 mg every other day; 100, 150, 200, 250 mg/day for 28 days treatment for 4 weeks in mild to moderate patients with VL and parasites (amastigotes) by Giemsa stained splenic aspirate (N=5/dose group) Dosages of ≥100 mg/day required for definite cure.
3089 (Phase 2) Varanasi and Muzaffarpur, India (*L. donovani*)	Open label, multi-center, randomized dose (100, 150, and 200 mg/day for 28 days) ranging study. The primary efficacy endpoint of the study was the rate of patients with definite parasitological cure 6 months after end of treatment. Response was assessed by splenic or bone marrow aspirate and clinical parameters. In two patients the respective examination was done after 5 instead of 6 months. These patients were re-assessed 7 months later to confirm the response. All the patients (n=15) at 100 mg/day dose and 16 at 150 mg/day dose were cured. The highest dose (200 mg/day) was not well tolerated although all 7 subjects were cured.
3109 (Phase 2) Muzaffarpur and Patna, India (*L. donovani*)	Multicenter, open-label, sequential group dose escalating trial in VL, confirmed by spleen aspirate, and with signs and symptoms compatible with VL A majority of the patients, in the PP population at 100 mg/day (29/30) and 150 mg/day (100%) dose were cured. Miltefosine was active not only in newly diagnosed patients but also in patients who did not respond to or had relapsed after treatment with pentavalent antimony or amphotericin B.
3127 (Phase 2) Varanasi and Muzaffarpur, India (*L. donovani*)	Single-center, open label, randomized trial with 3 parallel groups to assess the apparent and 6-months definite cure rates in patients with newly diagnosed or resistant/relapsing VL, confirmed by spleen or bone marrow aspirate, and with clinical symptoms. Miltefosine was administered at 100 mg/day for 2, 3, or 4 weeks with follow-up for 6 months after the end of treatment. The definite parasitological cure rates after treatment with miltefosine were 89% after 2 weeks treatment and 100% after a 3 or 4 weeks therapy.
3091 (Phase 2) Muzaffarpur, India (*L. donovani*)	Multicenter, open-label trial in children (2 - 11 years), with newly diagnosed or resistant/relapsing VL and presenting with clinical signs and symptoms compatible with VL. Patients were treated with 1.5 mg/kg/day (n=21) or 2.5 mg/kg/day (n=18) for 4 weeks. Follow-up was 6 months. Initial and final parasitological cure in all patients were similar. Regression of spleen size and resolution of fever was slightly faster in patients treated with 2.5 mg/kg/dose than the lower dose. All patients in whom prior therapy had failed to eradicate the infection were finally cured after treatment with miltefosine.
3206 (Phase 3) Muzaffarpur, Patna, and Varanasi, India (*L. donovani*)	Open-label, to evaluate the treatment schedule of miltefosine (2.5 mg/kg/day for 28 days) in children (2 to 11 years) with newly diagnosed or resistant/relapsing VL. Cure rates were about 95% at 6 months follow-up after the end of treatment; 3 of the 80 patients enrolled relapsed.
Z013a and Z013b (Phase 4) Outpatient trial in Bihar, India and Nepal (*L. donovani*)	Open label, single arm trial in 1055 patients with newly diagnosed or resistant/relapsing VL. Miltefosine was administered for 28 days at a daily dose of 50 mg to adults <25 kg and 100 mg to adults ≥25 kg; children (2-11 years) were administered 2.5 mg/kg/daily. In India, the rate of patients with final cure (>95%) in this out-patient trial was similar to the cure rate that had been achieved in hospitalized patients in the Phase 3 trial study 3154. It was assumed that the majority of patients with initial cure who did not appear for the post-treatment control visits did so because they were still free of disease. In Nepal, the cure rates were about 97% and final cure rates about 84%, including treatment of patients who were resistant to treatment with sodium stibogluconate.
Rahman *et al.*, 2009 (Phase 4) Bangladesh (*L. donovani*)	Single group trial in 977 patients with newly diagnosed or resistant/relapsing VL with and without splenomegaly, confirmed by a positive serological test (using freeze dried antigen) for Kala-azar (rK39 immunoreactivity). Patients were dosed with miltefosine as summarized above for Study Z013 to a target dose of 2.5 mg/kg/day for 28 days. Cure rates for the splenomegaly group and the no splenomegaly group were similar. About 92% of the subjects were initially cured and 74% and 85% in PP and evaluated population, respectively, at 6 month follow-up.
Z019 (Phase 4) Brazil (*L. chagasi*)	A dose ranging study in 43 subjects and miltefosine administered 100 mg/kg/day to adults and 2.5 mg/kg/day to children for 28 to 42 days. The cure rates were about 74% in adults and 43% in children. The 3 adult patients who relapsed were administered 2, 2 and 1.4 mg/kg/day of miltefosine. After 42 days of treatment at one site, the response rate was 68% in children.
CSR 1 (Phase 2) Europe (Spain, France, Italy, Portugal) (*L. infantum*)	Compassionate administration of miltefosine at a dose of 100 mg/kg/day to VL patients with (n=39) and without (n=5) underlying HIV infection and had multiple relapses after prior standard therapies. Initial responses in 25 of 39 (64%) patients, including clinical cure in 16 and definite improvement in 9 patients

*_Leishmania_ species based on epidemiology. Parasitological observations include examination of aspirates or smears after staining. In-patient trial unless specified otherwise. VL=visceral leishmaniasis

5.2. Mucosal leishmaniasis

The efficacy and safety of miltefosine was measured in patients with mucosal leishmaniasis in one study [Study Z022 (Bolivia)]. Based on epidemiological findings, Bolivia is known to be endemic for *L. braziliensis*.

5.2.1. Study Z022

This was a phase 2 randomized, active controlled trial to evaluate the effectiveness and tolerance of miltefosine (2.5 mg/kg/day for 28 days) in the treatment of 79 patients with mucosal leishmaniasis in Bolivia (Study report August 8, 2012; Soto *et al*., 2007[50]). The active control was pentavalent antimony; however, the trial was amended and intravenous amphotericin B (1 mg/kg amphotericin B every other day for a total of 45 injections over 3 months) was included as a comparator when the study team became aware that pentavalent antimony had been rejected as ineffective for mucosal leishmaniasis at this site. The applicant states that when the efficacy of oral miltefosine became apparent in initial patients, additional patients refused to be entered into an amphotericin B arm. Therefore, the final study design became an evaluation of 1 cohort of 79 patients administered miltefosine.

Study design

Inclusion criteria

- Age: >18 years.
- Male, and female (if accepted, contraceptive measures were agreed to be used during the time of the treatment and for 2 months after the last dose of the medicine).
- History of leishmaniasis: suggestive scar of previous cutaneous leishmaniasis.
- History of mucosal leishmaniasis: mucosal symptoms.
- Symptoms of mucosal leishmaniasis: erythema, edema, infiltration, erosion of the nares and/or nasal septum and/or epiglottis, uvula, or palate.
- Parasitology: *Leishmania* seen in cultures or in histopathological examination of lesion aspirates OR positive Montenegro test (skin test signifying *Leishmania* infection).
- History of treatment for leishmaniasis: no previous treatment for mucosal leishmaniasis, or if previously treated, the treatment must have been at least 6 months prior to this trial and symptoms must have progressed in the last 3 months.
- Informed consent: patients must have given written consent.
- Concomitant diseases: no clinically significant abnormalities on physical examination.
- Laboratory values: without clinically significant abnormalities in hematology, transaminases, alkaline phosphatase, total bilirubin, and creatinine.

Exclusion criteria

- Unable to comply with protocol: inability to complete the requirements of the study and to attend the follow-up visits.

The efficacy of miltefosine was evaluated by examining the nasal and oral mucosa of each patient at the beginning of therapy, the end of therapy, and at 2, 6, 9, and 12 months after the end of therapy. Subjects were assessed for clinical response at different visits; parasitological response was measured, if clinically indicated (Table 29).

[50] Soto J, Toledo J, Valda L, Balderrama M, Rea I, Parra R, Ardiles J, Soto P, Gomez A, Molleda F, Fuentelsaz C, Anders G, Sindermann H, Engel J, and Berman J. Treatment of Bolivian mucosal leishmaniasis with miltefosine. Clinical Infectious Diseases (2007) 44: 350–356.

Table 29: Study Z022 - Design and schedule of assessments

Procedure	Treatment period			Post-treatment follow-up				
	Day 0[a]	Day 14	Day 28	2 weeks	2 months	6 months	9 months	12 months
Consent	X							
History/physical exam	X		X	X	X	X	X	X
Vital signs	X	X	X	X	X	X	X	X
Parasitology	X		X	X[b]	X[b]	X[b]	X[b]	X[b]
Photograph lesions	X		X	X	X	X	X	X
Hematology[c]	X	X	X					
Chemistry[d]	X	X	X					
Urinanalysis	X		X					
Pregnancy test	X		X					
Treatment[e]	X	X	X					
Adverse events[f]	X	X	X					

[a] Day 0 refers to the screening period, which was typically 7 days (range 3-30 days).
[b] Post-treatment parasitology was to be performed if clinically needed.
[c] Hematology included white blood cell count with differential, platelet count hemoglobin.
[d] Chemistry included aspartate aminotransferase (AST), alanine aminotransferase (AST), total bilirubin, alkaline phosphatase, and creatinine.
[e] Treatment was daily for 28 days.
[f] AE were assessed twice weekly during treatment.

The mucosal leishmaniasis severity score was computed, at any assessment time, by adding the severity score (0 = none, 1 = mild, 2 = moderate, 3 = severe) for each of 4 pathological signs (erythema, edema, infiltration, and erosion) at each disease site. Efficacy responses were based on a change in the mucosal leishmaniasis severity score (Table 30) at 12 months compared with baseline.

Table 30: Study Z022 - Efficacy response definitions

Response	Change in ML Severity Score Compared with Baseline
cured	≥ 90% improvement
improved	50 to < 90% improvement
not changed	25% worsening to < 50% improvement
worsened	< 25% worsening
presumptive failure	discontinued follow-up because cure at 12 months was unlikely

Parasitological measurements:
Parasitological measurements at screening included Giemsa staining, culture, and/or isoenzyme electrophoresis of aspirates and/or biopsy specimens. Specimens were sent to (b) (4)

(b) (4) The intradermal skin test for *Leishmania* infection ("Montenegro test") was performed on patients by the (b) (4) staff.

It appears that up to 3 aspirations were performed for parasitological measurement before treatment; 1 lesion on each patient was aspirated to visualize parasites by Giemsa staining. If no parasites were visualized, a second and, if necessary, a third aspiration was performed. If the third aspiration did not reveal parasites, a biopsy sample was obtained for staining. The aspirate was also cultured. When culture results were positive, the parasites were speciated by isoenzyme electrophoresis. For patients for whom aspiration and biopsy yielded negative results, parasitological diagnosis was based on a positive leishmanin skin test result and the presence of a scar that signified prior cutaneous disease.

Results

Every patient was stated to be positive by one of the 3 measures of *Leishmania* infection at screening (Table 31); 30 patients had smears in which *Leishmania* amastigotes were identified by Giemsa staining, and 8 patients had biopsies containing cultured parasites. Almost all patients (74/79 = 94%) were skin test positive. Seven of the 10 biopsies that contained parasites were successfully cultured; all cultured parasites were stated to be *L. braziliensis* by isoenzyme electrophoresis.

Table 31: Study Z022 - Parasitological findings at screening in all subjects

	Skin test			Smear/stain			Biopsy/culture	
Subjects N (%)	Negative N (%)	Positive N (%)	Not done or missing N (%)	Negative N (%)	Positive N (%)	Not done or missing N (%)	Negative N (%)	Positive N (%)
79 (100)	2 (3)	74 (94)	3 (4)	41 (52)	30 (38)	8 (10)	1 (1)	10 (13)

There was some discrepancy in the number of subjects reported to be positive at screening by culture/biopsy results in the publication (Soto *et al*., 2007).[50] In the publication, authors reported 64% (n=50) as parasite positive by culture of aspirates or biopsy specimens and state that this percentage of parasitologically positive patients is consistent with the difficulty of finding parasites in mucosal disease.

The modified (m) ITT population included 79 subjects who were exposed to study medication and for whom at least one follow-up documentations of any efficacy data was available. The PP population included 76 subjects who fulfilled the selection criteria and who received the scheduled trial medication on at least 90% of the planned treatment days and who were assessed after 12 months.

Mean mucosal leishmaniasis severity scores steadily decreased approximately 5-fold (from10 to 2) over the 13 months of the trial in patients treated with miltefosine. Approximately 65% of the subjects were cured in the PP population (Table 32). The total severity score correlated with clinical response. Patients that were cured had a lower severity score at baseline compared to those with a higher severity score and there was a trend toward lack of cure for distal disease versus solely proximal disease (Table 32). However, there was no correlation between parasite-positivity at baseline and clinical response. For all patients, mucosal scores decreased by 2 months post-treatment.

The cure rate for a small number of subjects (n=14) administered amphotericin B (45 mg/kg over 90 days) was 50%.

Table 32: Study Z022 - Clinical response at month 12

Clinical response in PP and mITT population

Clinical Response	PPS n=76		mITT n=79	
	N	%	N	%
Cured	49	64.5	49	62.0
Improved	16	21.1	16	20.3
No Change	6	7.9	6	7.6
Worsened	1	1.3	1	1.3
Presumptive Failure	4	5.3	4	5.1
Not Evaluable			3	3.8

Clinical response by baseline line characteristics in PP population

Characteristic	Cured	Improved	No change/ Worsened/ Presumptive Failure	p-value
Number of Subjects	49	16	11	
Age, years	39.3±16.4	39.3±16.1	40.1±17.0	0.9884[a]
Weight, kg	58.9±8.7	58.5±11.0	53.6±5.9	0.2048[a]
Male Sex	33 (67.3)	15 (93.8)	8 (72.7)	0.1140[b]
Parasitologically positive	24 (52.2)	5 (41.7)	4 (36.4)	0.5740[b]
Number of ML sites	1.7±0.8	2.4±1.2	2.1±1.4	0.0260[a]
Distal disease type	22 (44.9)	12 (75.0)	5 (45.5)	0.1027[b]
Proximal disease type	27 (55.1)	4 (25.0)	6 (54.5)	0.1027[b]
ML severity score as screening	7.5±4.6	16.1±9.1	13.8±13.5	0.0002[a]

[a] p-value by ANOVA.
[b] p-value by chi-square analysis.

Comments:

- *Cultures results were available from 8 patients and all were stated to be L. braziliensis by isoenzyme analysis. About 92% percent of Bolivian leishmaniasis is known to be caused by L. braziliensis.*
- *Mucosal scores decreased by approximately 60% by 2 months post-treatment; 49 (65%) of the 76 in the PP population were "cured" by the definition of ≥ 90% reduction in the mucosal severity score at the 12-month follow-up.*
- *The severity score prior to treatment appeared to have an effect on response to treatment. Patients that were cured had a lower severity score at baseline compared to those with a higher severity score and there was a trend toward lack of cure for distal disease versus solely proximal disease. However, there was no correlation between parasite-positivity at baseline and clinical response.*

5.3. Cutaneous leishmaniasis

The efficacy and safety of miltefosine was measured in patients with cutaneous leishmaniasis in one pivotal study (Study 3168 in Guatemala and Colombia) and 5 supportive studies [Studies Z020a (Brazil) and Z020b (Brazil), Study Soto (Bolivia), Study 3092 (Colombia), and Study Z026 in Kabul, Afghanistan]. Of these, 4 studies were conducted in South America (Colombia, Guatemala, Brazil, and Bolivia) known to be endemic for the New World *Leishmania* species and one in Kabul known to be endemic for the Old World *Leishmania* species.

5.3.1. Study 3168

This was a randomized, placebo-controlled, double blind, multi-center trial with 2 parallel groups in 133 patients (89 in the miltefosine arm and 44 in the placebo arm) with cutaneous leishmaniasis from two sites (Colombia and Guatemala) in South America (Report no D-18506 / 2200000056-01; Clinical study report addendum, April 19, 2013; Soto *et al*., 2004[51]). In Colombia, *L. panamensis, L. braziliensis, L chagasi*, *L. guyanensis,* and *L. amazonensis* are prevalent whereas in Guatemala *L. braziliensis* and *L. mexicana* species are known to be common. The study was performed between 2000 and 2002. Subjects were treated for 28 days with either placebo or miltefosine at a dose of 100 – 150 mg/day to achieve target of 2.5 mg/kg/day.

Primary objective was to demonstrate superiority of miltefosine over placebo at 2 weeks (partial/ apparent cure) after the last dose followed by definite cure at 6 months.

Secondary objectives

• Assessment of definite cure rate (after 6 months follow up).
• Characterization of the safety.

Study design

Inclusion criteria

- Males and females ≥ 12 years.
- Newly diagnosed (within 4 weeks of start of treatment) or resistant/relapsing cutaneous leishmaniasis without mucosal involvement, parasitologically confirmed.
- Indicator lesions: at least one skin ulcer or inflammatory induration with positive parasitology (minimum area: 50 mm^2).

Exclusion criteria

- Concomitant treatment with other anti-leishmanial drugs.
- Prior anti-leishmanial therapy within the last 4 weeks or pretreated lesions still improving.
- Any condition which compromised the ability to comply with the study procedures.
- Participation in any other clinical trial.
- Safety issues such as lactation, pregnancy.

Evaluations

Patients were evaluated for clinical features and laboratory parameters including parasitological measurements at baseline and different time intervals after initiation of therapy (Table 33). Clinical evaluations included measuring size and induration of each lesion. Lesions for which

[51] Soto J, Arana B.A, Toledo J, Rizzo N, Vega JC, Diaz A, Luz M, Gutierrez P, Arboleda M, Berman JD, Junge K, Engel J, and Sindermann H. Miltefosine for New World cutaneous Leishmaniasis. Clin Infectious Dis (2004) 38: 1266-1272.

there was incomplete re-epithelialization of the ulcer or incomplete elimination of induration underwent repeated parasitological investigation. Follow up parasitological measurements were performed at the discretion of the investigator.

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Table 33: Study 3168 - Time schedule

FLOW CHART							
	Visit	1	2	3[g]	4	5	6[i]
Assessment	Day	before[a]	weekly during treatment (day 7,14,21)	Day 28 or end of treatm.	2 weeks after end of treatm.	2 month after end of treatm.	6 month after end of treatm.
Informed consent		X					
Randomization		X					
Medical History		X					
History of leishman.		X					
Stool parasitology / malaria test [b]		X					
Pregnancy test (females of child-bearing potential)[c]		X		X			
HIV test		X					
Efficacy - leish. parasitology: stain/culture/PCR[d]		X			(X)	(X)	(X)
- size / grade of epithelialization / area of infiltration		X			X	X	X
- photograph[e]		X			X	X	X
Laboratory[f] - Haemogram		X	X	X		X	X
- Chemistry		X	X	X		X	X
- Urinalysis		X		X			
Vital parameters		X	X	X		X	X
Ophthalmology		X		X			X
Medication			daily				
Global ass. tolerab.				X			
Concomit. diseases		X	monitoring of concomit. diseases				
Adverse events[h]		X	documentation as occurring				
Spermiogram (male patients)		X			X		X

a Within 7 days before 1st dose; parasitological examination of skin lesions valid within a period of 4 weeks prior to treatment start.
b Stool parasitology only required in case of clinically suspected gastrointestinal infection.
c Screening assessment performed in the first half of menstrual cycle (≤ day 10 of cycle): in case of negative pregnancy test prior to randomization, treatment could be started without waiting period. Screening assessment performed in the second half of menstrual cycle (> day 10): pregnancy test to be followed by a two weeks waiting period during which adequate contraceptive measures were to be used. After a waiting period a confirmatory pregnancy test was to be done before randomization.
d Parasitology (stain, culture, PCR) to be done from each lesion which was not completely epithelialized or which showed inflammatory induration at screening (diagnosis). At 2 weeks after end of treatment and during follow up it was the investigator's decision whether a parasitological examination would be performed for lesions which were not completely healed. Samples from positive cultures at screening assessment were also used for leishmania speciation
e A standardized photograph (according to the method approved by the center) was to be taken from each lesion.
f Additional examinations were to be performed as medically indicated. In case of CTC grade 3/4 toxicity a confirmation measurement was to be done on the next day
g A full assessment should be done whenever treatment is discontinued. The reason(s) for discontinuation was to be documented. This also applied to patients withdrawing from the study for whatever reason.
h Follow up of adverse events persisting at the end of treatment according to chapter 8.5 of protocol.
i The final evaluation was scheduled 6 months after end of treatment. In a patient with a relapse, the final evaluation was to be performed when the relapse was diagnosed.

Parasitological measurements:

The lesion specimens for parasitological measurements were collected by one or more of the following methods:

- Slit skin smear
- Aspirate
- Biopsy

Identification of amastigotes was performed by microscopic examination of either Giemsa stained smears or cultures of motile promastigotes from the aspirate and/or biopsy of a lesion. Due to technical difficulties (pain to the patient, bacterial contamination) and cost, some amendments were made to the protocol as preparation of culture from each lesion was not feasible. In Colombia, parasites after culture were processed for identification of *Leishmania* species by an immunofluorescent assay (IFA) using monoclonal antibodies by a method described elsewhere (Chico *et al*., 1995[52]) in a central laboratory (b) (4) . In Guatemala, *Leishmania* species was identified using cultures or clinical samples preserved in absolute alcohol by polymerase chain reaction (PCR).[53, 54] The laboratory where testing by PCR was performed was not specified. The details of the method or performance characteristics of the IFA or the PCR assay were not included in the study report. The applicant refers to a publication by Soto *et al*. (2004)[51], for details of the methods and the performance characteristics of the assays. A summary of the information available from these publications is briefly discussed below:

Immunofluorescent assay:

The study by Chico *et al.* (1995),[52] reported evaluation of a direct IFA using a specific monoclonal antibody (83J3D2) against the *Leishmania* genus for routine diagnosis of cutaneous leishmaniasis in Ecuador. The monoclonal antibody 83J3D2 recognizes a dominant antigen common to promastigotes of isolates from 3 major species and 5 subspecies of New World cutaneous leishmaniasis. The authors refer to another publication for details of the method (Grogl *et al*., 1989[55]) which was not available for review despite best efforts that included request from the Armed Forces library through the FDA library).

Comment:

According to Chico et al., the monoclonal antibodies can aid in identifying Leishmania genus but not Leishmania species as it recognizes a dominant antigen common to promastigotes of isolates from 3 major species and 5 subspecies of New World cutaneous leishmaniasis. Therefore, the assay is not useful for identification of any Leishmania species.

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[52] Chico ME, Guderian RH, Cooper PJ, Armijos R, and Grogl M. Evaluation of a direct immunofluorescent antibody (DIFMA) test using *Leishmania* genus–specific monoclonal antibody in the routine diagnosis of cutaneous leishmaniasis. Rev Soc Bras Med Trop (1995) 28:99–103.

[53] Noyes HA, Belli AA, and Miangon R. Appraisal of various random amplified polymorphic DNA–polymerase chain reaction primers for *Leishmania* identification. Am J Trop Med Hyg (1996) 55:98–105.

[54] Noyes HA, Reyburn H, Bailey JW, and Smith D. A nested-PCR–based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. J Clin Microbiol (1998) 36:2877–2881.

[55] Grogl M, Milhous WK, Martin RK, and Kyle DE. Kits for the diagnosis of cutaneous leishmaniasis in field laboratories. Proceedings of the Society of Armed Forces Laboratory Science (1989) 18: 22.

Polymerase chain reaction:

The study by Noyes *et al*. (1996[53]) reported testing of 11 isolates/strains of *Leishmania* species [*L. (V.) braziliensis* (n=4), *L. (V.) guyanensis* (n=1), *L. (V.) panamensis* (n=3), *L. peruviana* (n=1), and *L. (Viannia)* species (n=2)] conducted at (b) (4) . Briefly, DNA was prepared from promastigotes from six strains isolated in Nicaragua from cases of American cutaneous leishmaniasis and typed for isoenzyme pattern by biochemical tests and restriction length fragment polymorphisms (RFLPs) and from five reference strains cultured *in vitro* in the medium suggested by the supplier of the parasites. The different random amplified polymorphic DNA (RAPD)-PCR primers (n=28) included for testing (Table 34A) were same as previously assessed, by the authors, for sensitivity, specificity, and reliability of distinguishing each of four closely related New World *Leishmania* species. The degree of relatedness between species was quantified and estimates were made of the accuracy and precision of the determinations. Thirteen primers showed more than five bands and generated unique patterns for each species and so could be used for identification (Table 34B); however, no single primer generated a major unique product for each of the four species. Based on testing of 11 strains that include 5 species of *Leishmania*, primers that reliably separated pairs of species were identified (Table 34B).

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Table 34: PCR assay – Primers used and species identified

A: Polymerase chain reaction primers and thermal cycling programs

Primer[a,c]	Sequence	Amplification*	Thermal cycles†
M13	GTAAAACGACGGCCAGT	++	1
AB1-01	GTTTCGCTCC	++	2
AB1-02	TGATCCCTGG	–	2
AB1-03	CATCCCCCTG	++	2
AB1-04	GGACTGGAGT	++	2
AB1-03	CATCCCCCTG	+	2
AB1-04	GGACTGGAGT	++	2
AB1-05	TGCGCCCTTC	–	2
AB1-06	TGCTCTGCCC	+	2
AB1-07	GGTGACGCAG	++	2
AB1-08	GTCCACACGG	–	2
AB1-09	TGGGGGACTC	++	2
AB1-10	CTGCTGGGAC	++	2
AB1-12	CCTTGACGCA	++	2
AB1-13	TTCCCCCGCT	++	2
AB1-14	TTCCCCCGCT	++	2
AB1-15	GGAGGGTGTT	++	2
AB1-16	TTTGCCCGGA	+	2
AB1-17	AGGGAACGAG	–	2
AB1-18	CCACAGCAGT	++	2
AB1-19	ACCCCCGAAG	++	2
AB1-20	GGACCCTTAC	–	2
A1[16]	CAGGCCCTTC	++	2
A4[16]	AATCGGGCTG	++	2
A7[16]	GAAACGGGTG	++	2
A8[16]	GTGACGTAGG	++	2
3301[33]	TCGTAGCCAA	++	3
ILO525[17]	CGGACGTCGC	++	2

* – = none; + = weak; ++ = strong.
† 1 = two cycles at 94°C for 2 min, 40°C for 5 min, followed by five cycles at 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min; 2 = 35 cycles at 94°C for 30 sec, 36°C for 1 min, and 72°C for 2 min; 3 = 30 cycles at 94°C for 1 min, 40°C for 1.5 min, and 72°C for 2 min.

B: Random amplified polymorphic DNA-polymerase chain reaction primers that discriminate between *L. Viannia* species*

L. braziliensis	—			
L. panamensis	AB104	—		
L. guyanensis	AB118	AB109	—	
L. peruviana	A8	AB118	AB113	—
	L. braziliensis	*L. panamensis*	*L. guyanensis*	*L. peruviana*

* The primers that are indicated against pairs of species generate at least one major unique product in each member of the pair.

The sensitivity of the RAPD-PCR was lower (requiring 1-10 ng of DNA, optimum 5 ng) than that reported for the conventional PCR primed by kDNA sequences (0.14 pg); assuming 4 x $l0^7$ bp for the haploid *Leishmania* genome, 10^4 parasites were reliably detectable by RAPD whereas the conventional PCR could detect a single parasite.

The authors state that RAPD finger prints should be carefully interpreted. In particular, the size range of products may vary with the quantity and quality of the DNA; the absence of a particular product in a size range where no other products are visible should not be interpreted as evidence of the absence of the corresponding template DNA. The size range of products may vary from batch to batch of DNA. Changes in annealing temperature by even 1^oC can alter the sensitivity of RAPD. Alteration in the concentrations of primers, *Taq* polymerase, and dNTPs used can vary the number of products generated. Since the accuracy of even the best thermalcyclers is not better than $\pm 0.5^oC$, results are liable to vary with the cycler used, making inter-laboratory comparisons of fingerprint patterns difficult.

Another study (Noyes *et al*. 1998[54]), reported testing by nested PCR on 20 samples from patients from the Timargara refugee camp, Pakistan. Samples were collected by scraping out a small

amount of tissue with a scalpel from an incision at the edge of the lesion; the tissue was smeared on one microscope slide and placed in a tube of 4 M guanidine thiocyanate, in which the sample was stable for at least 1 month. DNA for PCR was prepared by being bound to silica in the presence of 6 M guanidine thiocyanate, washed in guanidine thiocyanate, ethanol, and acetone and then eluted. PCR products of the size expected for *L. tropica* were obtained from 15 of the 20 samples in at least one of three replicate reactions. The negative samples were from lesions that had been treated with glucantime or were over 6 months old, in which parasites were known to be scarce. The authors state that this test is now in routine use for the detection and identification of *Leishmania* parasites in their clinical laboratory. Fingerprints produced by restriction digests of the PCR products were defined as complex or simple. There were no reproducible differences between the complex restriction patterns of the kinetoplast DNA of any of the parasites from Timargara camp with HaeIII and HpaII; however, the simple fingerprints were very variable and were interpreted as being the product of PCR on a limited subset of minicircle classes, and consequently, it was thought that the variation was determined by the particular minicircle classes that had been represented in the template. The authors state that homogeneity of the complex fingerprints suggests that the present epidemic of cutaneous leishmaniasis in Timargara camp may be due to the spread of a single clone of *L. tropica*. It is of note that the complex finger patterns prepared from the clinical samples were not fully reproducible on replicate DNA preparations from the same clinical sample.

Overall and parasitological response

A lesion was defined as a treatment failure if it enlarged by 50% or was positive for parasites between 2 weeks to 6 months after the end of therapy, relapsed (enlarged) after previously diminishing in size, or did not completely re-epithelialize by 6 months after the end of therapy. Appearance of a new lesion from which *Leishmania* could be demonstrated was also a criterion for failure (for details see Table 35).

Primary efficacy endpoint*: final (6 month) clinical response*

Definite cure: Apparent cure or partial cure at 2 weeks after the last dose followed by definitive cure at 6 months.

Clinical failure: Failure at 2 weeks or failure at 6 months.

Not assessable***:*** Not assessable at 6 months.

Table 35: Study 3168 - Definitions of parasitological and overall response

Parasitological Response	Overall Response
Presence of parasites (treatment failure): Based on a positive finding with one out of the three methods (smear / aspirate / biopsy) in one lesion. **Absence** of parasites: Based on a negative findings with at least two out of the three methods in each lesion (smear / aspirate / biopsy), that was not clinically cured. **Treatment failure:** A positive parasitology at or after week 2 after end of treatment**.**	**Responses at 2 Weeks Follow-up*:** ***Apparent cure*** Complete epithelialization of all ulcers, **and** complete disappearance of inflammatory induration from all lesions 2 weeks after end of treatment. ***Partial cure***** Incomplete epithelialization or incomplete regression of inflammatory induration of one or more lesions, **and** • No more than 50% enlargement of previously documented lesions, **and** • Absence of parasites (if tested for), **and** • No appearance of new lesions. ***Clinical failure*** Lack of achieving partial cure, defined as: • 50% enlargement of the total lesion area, **or** • Presence of parasites, **or** • New lesion. ***Not assessable*** Not seen at this time period **Responses at 6 Months Follow-up:** ***Definite cure*** • Complete epithelialization of all ulcers. • Complete disappearance of inflammatory induration from all lesions. • No new lesions between 2 weeks and 6 months. • No 50% enlargement of lesion between 2 weeks and 6 months. • No relapse. • No new *Leishmania*-positive lesion could appear. **Failure** Not achieve Definitive cure. **Not assessable** Not seen at this time period**.** If no parasitology was done at 2 weeks after end of treatment and /or during follow up, the evaluation of treatment failure was based on clinical criteria only. A positive parasitology at or week 2 after end of treatment was defined a treatment failure.

* Comment: Patients who failed at 2 weeks according to these criteria may have been followed at later time periods, but if a patient failed at 2 weeks, the Final (6 month) Clinical Response for that patient was “clinical failure”.
***If no parasitology was done 2 weeks after end of treatment the evaluation of partial cure is based on clinical parameters only. A positive parasitology at or week 2 after end of treatment was defined a treatment failure.

Results

At baseline, all patients were parasitologically positive by Giemsa-stained smears prepared from a slit-skin of the lesion or by culturing from lesion aspirates. The ITT population included all randomized and exposed patients and the PP population included all patients who fulfilled the selection criteria and received the scheduled trial medication on at least 90% of the planned treatment days and were assessed at least for apparent cure.

Confirmed diagnosis of cutaneous leishmaniasis dated back between 3 and 47 days (inclusion criteria was 3-28 days) before start of study treatment. At baseline, patients presented with up to

10 skin lesion; the number of lesions varied from 1 to 8 in Colombia and 1 to 10 in Guatemala. About 60% of the patients, in either arm, had one skin lesion. Leishmaniasis was newly diagnosed in 34/44 (77.3%) placebo patients and in 77/89 (86.5%) miltefosine patients. Ten of the 44 (22.7%) placebo patients and 13 (14.6%) of the miltefosine patients had a pretreatment recorded for leishmaniasis (including one miltefosine-treated patient, who was classified as newly diagnosed). The most frequently used drug was Glucantime.

Overall, the results show that miltefosine was effective in the treatment of cutaneous leishmaniasis in both ITT and PP population at 2 weeks and 6 months after end of therapy (Table 36).

Table 36: Study 3168 – Summary of clinical and parasitological responses

Treatment group/ site	**2 weeks after end of therapy n/N %**			**Follow up at 6 months n/N %**		
	Clinical success (Apparent cure or partial cure)	Proven parasitological eradication n negative/ n tested)	Presumed parasitological eradication n cured/ n not tested	Clinical success (Final cure)	Final cure with proven parasitological eradication n negative/ n tested	Final cure with presumed parasitological eradication n cured/ n not tested
ITT - Miltefosine						
Colombia[a]	45/49 (92%)	8/9 (89%)	37/40 (92%)	40/49 (82%)	9/11 (82%)	31/38 (82%)
Guatemala[b]	24/40 (60%)	11/23 (48%)	13/17 (76%)	19/40 (48%)	13/25 (52%)	6/15 (40%)
Total both sites	69/89 (78%)	19/32 (59%)	50/57 (88%)	59/89 (66%)	22/36 (61%)	37/53 (70%)
ITT- Placebo						
Colombia	10/24 (42%)	8/21 (38%)	2/3 (67%)	9/24 (38%)	8/22 (36%)	1/2 (50%)
Guatemala	7/20 (35%)	2/14 (14%)	5/6 (83%)	4/20 (20%)	2/16 (12%)	2/4 (50%)
Total both sites	17/44 (39%)	10/35 (29%)	7/9 (78%)	13/44 (30%)	10/38 (26%)	3/6 (50%)
Total both groups	86/133 (65%)	29/67 (43%)	57/66 (86%)	72/133 (54%)	32/74 (43%)	40/59 (68%)
PP - Miltefosine						
Colombia	45/47 (96%)	8/9 (89%)	37/38 (97%)	40/47 (85%)	9/11 (82%)	31/36 (86%)
Guatemala	24/38 (63%)	11/23 (48%)	13/15 (87%)	19/38 (50%)	13/25 (52%)	6/13 (46%)
Total both sites	69/85 (81%)	19/32 (86%)	50/53 (94%)	59/85 (69%)	22/36 (61%)	37/49 (76%)
PP - Placebo						
Colombia	10/24 (42%)	8/21 (38%)	2/3 (67%)	9/24 (38%)	8/22 (36%)	1/2 (50%)
Guatemala	7/18 (39%)	2/13 (15%)	5/5 (100%)	4/18 (22%)	2/15 (13%)	2/3 (67%)
Total both sites	17/42 (40%)	10/34 (29%)	7/8 (87%)	13/42 (31%)	10/37 (27%)	3/5 (60%)
Total both groups	86/127 (68%)	29/56 (52%)	57/61 (93%)	72/127 (57%)	32/73 (44%)	40/54 (74%)

[a] Speciation not routinely performed. All parasites from this site are *L v panamensis* by epidemiology.
[b] Differentiation between *L. v braziliensis* and *L. mexicana* was performed by academics and patient-level data is not available.

The cure rates were higher in the patients from Colombia (Center 1) treated with miltefosine compared to those from Guatemala (Center 2) (Tables 36 and 37). Apart from a difference in the percentage of final cures, there seemed to be a difference between centers (countries) in the time

course of response onset, i.e., the time to healing of the skin lesions. Patients in Center 2, compared to patients in Center 1, showed a lower rate of spontaneous healing in the post-treatment period in addition to lower cure rates.

Table 37: Study 3168 - Primary efficacy endpoint (definitive cure) at 6 months post-treatment

	Placebo	**Miltefosine**
Definite cure (ITT)		
Center 1 (Colombia)	9/24 (37.5%)	40/49 (81.6%)*
Center 2 (Guatemala)	4/20 (20.0%)**	19/40 (47.5%)
Total	13/44 (29.6%)	59/89 (66.3%)
PP cure		
Center 1 (Colombia)	9/24 (37.5%)	40/47 (85.1%)
Center 2 (Guatemala)	4/18 (22.2%)	19/38 (50%)
Total	13/42 (31.0%)	59/85 (69.4%)

The applicant did not include any speciation data in the study report. However, the publication by Soto *et al*. (2004[51]) does show the speciation data by immunofluorescent assay and PCR. In Colombia, cultures of 7 baseline lesion aspirates were speciated by monoclonal antibody binding and immunofluorescent assay; all 7 parasites were stated to be *L. (V.) panamensis*; however, the cure rate in these 7 patients was not shown.

In Guatemala, 46 of the 60 infecting parasites were speciated by PCR; 63% of the speciated parasites were *L. (V.) braziliensis* and 37% were *L. (L). mexicana* (Table 38). The cure rate for all patients with *L. (V.) braziliensis* was lower (33%) than the cure rate of *L. (L.) mexicana* (64%). The authors (Soto *et al.,* 2004[51]) conclude that miltefosine is a useful oral agent against cutaneous leishmaniasis due to *L. (V.). panamensis* in Colombia but not against leishmaniasis due to *L. (V.) braziliensis* or *L. (L). mexicana* in Guatemala.

Table 38: Study 3168 - Infecting *Leishmania* species infecting Guatemalan patients with cutaneous leishmaniasis.

Source: Soto *et al*., 2004[51]

Comments:

- *Initial parasitological diagnosis and follow-up for parasite clearance was made by visualizing parasites in Giemsa-stained smears of a slit-skin lesion or by culturing from lesion aspirates; all patients were parasitologically positive at the time of enrollment by one of these two methods.*
- *In Colombia, Leishmania species was identified by IFA. In Guatemala, Leishmania species was identified by PCR. The applicant cross-referenced publications for details of the method and performance characteristics of the assays. However, information in the publication was insufficient for review and the authors cross-referenced other references. Additionally, all*

the isolates from patients in the clinical trial were tested in laboratories other than those reported in the cited publications reporting testing of strains/isolates by IFA and PCR methods. Also, cure rates by Leishmania species were not included in the datasets.

Upon request from the Division for details of the methods and performance characteristics of the assays, in the laboratory, where testing of the clinical trial isolates was performed as well as the speciation data for the clinical trial isolates, the applicant stated that these assays were performed by academics and do not meet the standard of FDA regulations; also, patient-level data, by species, were not available. Therefore, data were reported for all species together rather than by individual species.

It is noteworthy that the references provided for the IFA test support the identification of Leishmania genus but not Leishmania species as the monoclonal antibody used in the test recognizes a dominant antigen common to promastigotes of isolates from 3 major species and 5 subspecies of Leishmania known to cause New World cutaneous leishmaniasis. Therefore, the assay is useful for identification of Leishmania genus but not for identification of any Leishmania species.

The laboratory in Guatemala where speciation of clinical isolates by PCR was performed was not specified. The number of strains tested for standardization of the assay (as listed in the publication) was small for any Leishmania species [L. (V.) braziliensis (n=4), L. (V.) guyanensis (n=1), L. (V.) panamensis (n=3), L. (V.) peruviana (n=1), and L. (Viannia) species (n=2)]. Additionally, several factors can affect intra- and inter-laboratory variability that include

- *size range of products that may vary with the quantity and quality of the DNA; the absence of a particular product in a size range where no other products are visible should not be interpreted as evidence of the absence of the corresponding template DNA. The size range of products may vary from batch to batch of DNA.*
- *annealing temperature: change in annealing temperature by even 1^oC can alter the sensitivity of RAPD.*
- *alteration in the concentrations of primers, Taq polymerase, and dNTPs used can vary the number of products generated.*
- *Thermacyclers: since the accuracy of even the best thermalcyclers is not better than ± 0.5^oC, results are liable to vary with the cycler used, making inter-laboratory comparisons of fingerprint patterns difficult.*

In the absence of review of details of the methods and performance characteristics of the IFA or PCR assays, the results were analyzed by region and not Leishmania species identified. Overall, the results suggest miltefosine to be effective for the treatment of cutaneous leshmaniasis in Colombia known to be endemic for L. panamensis, L. braziliensis, L. chagasi, L. guyanensis, and L. amazonensis; in patients from Guatemala, known to be endemic for L. braziliensis and L. mexicana, the cure rates were lower.

5.3.2. Study Z020a

This was a phase 3 randomized, open-label, controlled clinical trial to evaluate the efficacy and safety of miltefosine (2.5 mg/kg/day for 28 days) compared to parenteral (intravenous or intramuscular) meglumine antimonium (Glucantime®; 20 mg/kg/day for 20 days) for the treatment of cutaneous leishmaniasis in Manaus, Brazil (Study report dated August 7, 2012; Chruscial-Talhari *et al*., 2001[56]). This region is known to be endemic for *L. guyanensis.* A majority of the participants (98%) were infected patients from two cutaneous leishmaniasis endemic areas (Presidente Figueiredo and Rio Preto da Eva) located ~100 km from the city of Manaus.

Study design

Inclusion criteria

- Male and female subjects aged 2 to 65 years with a recent (duration not specified) diagnosis of cutaneous leishmaniasis.
- No previous treatment for leishmaniasis.
- Presence of at least 1 typical ulcerated lesion (round lesion, granulomatous bottom, elevated and indurated edges).
- Presence of a maximum of 5 lesions.
- Diameter of lesions between 1 and 5 cm.
- Clinical evolution of the disease not exceeding 3 months.
- Subjects had to have *Leishmania* parasites visible in a Giemsa smear of lesion aspiration biopsy, or smear, and speciation by PCR.

Exclusion criteria

- AST ≥3 times the upper limit of normal (ULN) of the reference range.
- ALT ≥3 times the ULN of the reference range.
- Alkaline phosphatase ≥3 times the ULN of the reference range.
- Total bilirubin ≥2 times the ULN of the reference range.
- Serum creatinine or BUN ≥1.5 times the ULN of the reference ranges.
- Evidence of serious underlying disease (cardiac, renal, liver, pulmonary, infectious, etc).
- Subjects under beta-blocker therapy (mainly sotalol and propranolol) and anti-arrhitmic drugs such as amiodarone and quinidine.
- Subjects with immunodeficiency or HIV antibodies.
- Pregnant or breast feeding women.
- History of prior treatment for leishmaniasis.
- Women of childbearing potential that did not accept the need of using an efficacious contraceptive method during the whole treatment period and for 3 additional months after the end of the study.
- Children with difficulty to swallow capsules.
- Serious concomitant infection other than cutaneous leishmaniasis (this includes evidence of other conditions associated to splenomegaly such as schistosomiasis).
- Severe protein and/or caloric malnutrition.
- Any decompensated or non-controlled condition such as active tuberculosis, malignant

[56] Chrusciak-Talhari A, Dietze R, Chrusciak-Talhari C, da Silva RM, Yamashita EPG, de Oliverra Penna G, Machado PRL, and Talhari S. 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. Am J Trop Med Hyg (2011) 84: 255–260.

disease, serious malaria, HIV, hanseniasis, systemic fungal disease (histoplasmosis, paracoccidioidomycosis), hepatitis B, hepatitis C, or any other infecto-contagious disease.
- Any condition that compromised the ability to comply with study procedures.
- Lack of capacity or willingness to sign the informed consent (subject and/or parents/legal representative).
- Lack of availability for visits/study procedures.

Screening for study eligibility was conducted over a 7 day period with the exception of the parasitology for leishmaniasis which was valid for a period of 4-weeks before randomization. Study drugs were administered daily on Days 1 to 28 for miltefosine and on Days 1 to 20 for Glucantime®. Subjects were followed for efficacy and safety for 6 months at 5 study visits following completion of treatment (Table 39).

The number and locations of cutaneous lesions was recorded at baseline. Each lesion was measured; the length and width of the ulcer in mm and the area of infiltration (indurated and erythematous border surrounding the ulcer) was recorded at the linear dimensions of the infiltrated region from the edge of the ulcer until the appearance of normal skin. Lesions were photographed; however, the photographs were not available for review.

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Table 39: Study Z020a - Procedures and schedule

Assessment[a]	Visit	1[b]	2	3	4	5	6	7	8	9	10
	Day	Pre	Day 7	Day 14	Day 21	Day 28 or end of treat-ment	2 weeks after end of treat-ment	1 month after end of treat-ment	2 months after end of treat-ment	4 months after end of treat-ment	6 months after end of treat-ment
Informed consent		x									
Clinical history		x									
Leishmaniosis history		x									
Parasitology (stool/blood direct test for Malaria)[c]		x									
Pregnancy test (women of childbearing potential)		x[d]		x				x			
HIV rapid test		x									
Efficacy											
Leishmania species (PCR)[e]		x									
- Leishmania parasitology: stain/culture		x					x[f]	x[f]	x[f]	x[f]	x[f]
- size/grade of epithelization/ infiltration area		x	x	x	x	x	x	x	x	x	x
- photography[g]		x	x	x	x	x	x	x	x	x	x
Laboratory[h]											
- WBC, platelets, hemogobin		x	x	x	x	x	x[h]	x[h]			
- Biochemistry		x	x	x	x	x	x[h]	x[h]			
- Urinalysis		x				x	x[h]	x[h]			
- Electrocardiogram (subjects from control group)		x	x	x	x	x	x	x			
Vital signs and AEs[i]		x	x	x	x	x	x	x	x	x	x

[a] A complete assessment was performed whenever treatment was discontinued. Reason(s) for discontinuation were documented. This also applied to subjects that withdrawn of the study due any reason.
[b] Within 7 days before 1st dosage (except for parasitological tests of the lesions, that will be valid within the last 4 weeks).
[c] Test for malaria was performed exclusively in Manaus.
[d] Selection assessment performed within the first half of the menstrual cycle (<day 10 of the cycle): in case of a negative pregnancy test before the random distribution, treatment was initiated without a waiting period. Selection assessment performed within the second half of the menstrual cycle (>10 days): pregnancy test followed by a waiting period of two weeks, during which appropriate contraceptive measures were used. After the waiting period, a confirmatory pregnancy test was performed, before the randomization.
Laboratory parameters consisted of
-Hematology: white blood cells (WBC), hemoglobin, and platelets
-Serum biochemistry: sodium, potassium, BUN, creatinine, ALT, AST, alkaline phosphatase
-Parasitology of the stools
-HIV serology by ELISA or rapid test (Determine or Rapid Check)
- Urinalysis (blood and protein only)
-Pregnancy test via serum β-hCG
[e] *Leishmania* speciation was performed by PCR based on amplification of the repeated heat shock protein 70 genes (Garcia et al-2004). Obtained patterns were compared with those of reference strains of *L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) lainsoni*, *L. (L.) amazonensis*, and *Leishmania (V.) naiffi*.
[f] Optional, except in cases of clinical deterioration or relapse.
[g] A standardized photograph was taken (according to the approved method by the site) of each lesion.
[h] Tests will be performed after the end of therapy when clinically indicated. In case of liver enzymes increase, control tests were performed immediately the day after (for confirmation) and thereafter, weekly, until the value(s) of the liver enzyme(s) was within the normal reference range.
[i] At each week return for drug dispensation at the study site, subjects were monitored for vomitting, diarrhea, somnolence, motion sickness, anorexia myalgia, and arthralgia.

Parasitological measurements

Parasitological measurements were performed in [redacted](b) (4) laboratory at [redacted](b) (4) Specimens (scraping or biopsy) were obtained for microscopic examination to confirm the presence of *Leishmania* parasites.

Identification of *Leishmania* species was performed by a PCR assay and for this the applicant refers to a publication by Chrusciak-Talhari *et al.* (2011[56]) which is a published report of the

Study Z020a. Briefly, the amplification of the repeated heat shock protein 70 (hsp70) genes was followed by restriction fragment length polymorphism analysis (hsp70 PCR-restriction fragment length polymorphism - PCR-RFLP) on skin biopsies from enrolled subjects. Restriction patterns were resolved by electrophoresis performed in 3% gel agarose stained with ethidium bromide. Obtained patterns were compared with those of reference strains of *L.* (*V.*) *braziliensis* (MHOM/BZ/75/M2903), *L.* (*V.*) *guyanensis* (MHOM/BR/75/M4147), *L.* (*V.*) *lainsoni* (MHOM/PE/03/LH2443), (MHOM/PE/87/LC106), *L.* (*L.*) *amazonensis* (MHOM/BR/81/LTB16), and *L.* (*V.*) *naiffi* (MDAS/BR/78/M5210). The method used for identification of *Leishmania* species of the reference strains was not specified.

The details of the assay summarized by the applicant in the NDA submission are same as those in the publication[56] and were insufficient for review of the details of the method and its performance characteristics; additionally, the publication[56] cross-references another publication (Garcia *et al.*, 2004[57]) for the PCR assay that reports testing of 34 biopsy specimens from Bolivian patients in a laboratory in Belgium or Peru. However, the laboratory where testing of clinical trial isolates was performed was in Manaus, Brazil and is different from that reported by Garcia *et al.* (2004)[57]. Additionally, the details of the method and performance characteristics of the assay were insufficient for review.

Primary endpoint was definitive cure at month 6 post-treatment and **secondary endpoint** was apparent cure at 2 months post-treatment (see Table 40 for details of efficacy response definitions).[58]

Failure was defined as the lack of either initial (apparent) cure at month 2 or final (definitive) cure at month 6. In addition, other reasons for failure were residual lesions with presence of parasites in a Giemsa stained imprint, or appearance of any new lesions, or ≥ 50% enlargement of previously documented lesions at any time prior to 6 months. Ulcer re-epithelialization and loss of induration was synonymous with clinical cure and therefore was a direct (non-surrogate) parameter of disease status.

[57] Garcia L, Kindt A, Bermudez H, Llanos-Cuentas A, De Doncker S, Arevalo J, Tintaya KW, and Dujardin JC. Culture independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. J Clin Microbiol (2004) 42: 2294 –2297.

[58] There were some differences in the definitions of protocol specified primary and secondary endpoints with those in the publication. The applicant redefined the primary and secondary endpoints due to the incomplete definition of definitive cure in the protocol, which did not incorporate some of the criteria that would otherwise define the subject as a treatment failure (i.e., apparent cure); this definition of clinical cure was stated to be consistent with clinical practice.

Table 40: Study Z020a - Efficacy response definitions

Definitive Cure:	100% re-epithelializaton and loss of induration of all initial lesions at 2-months and at 6 months; no new lesions, residual lesions with parasites or ≥ 50% enlargement of a lesion prior to 6 months. If data were missing at 2-months but the 6 month data met the criteria for cure, then the subject was classified as having cured. Subjects who did not have an assessment at 6 months were considered treatment failures.
Apparent Cure:	Complete re-epithelization of all ulcers by 2 months after the end of the treatment.
Failure:	Not meeting the criteria for cure.
Lost to Follow-up:	Did not come to final study evaluation at 6 months

The definitive cure rates of the Glucantime group in adolescent/adults were compared to that of the miltefosine group in adolescent/adults by two-sided Fisher's Exact test. P-values < .05 were considered statistically significant. Analyses were performed using SAS version 9.2.

Note: Induration criteria were not used for apparent cure, in accord with the protocol. Loss of induration at early follow up time points is not normally specified in clinical practice.

Results

Of the 90 subjects evaluated, 60 were adolescent/adult aged 12-65 years and 30 were pediatric subjects aged 2-11 years, who received at least one dose of drug and constituted the mITT and safety population. The PP population was not defined. Microscopy to diagnose the "*Leishmania*" species was positive in all but one subject in whom it was not performed (Table 41). By PCR, 98% of all subjects were stated to be infected with *L. guyanensis*, which is known to be the predominant (>95%) *Leishmania* species in Manaus; there was one case each of *L. lainsoni* and *L. braziliensis* in the miltefosine treated group (Table 41). The patient infected with *L. lainsoni* (miltefosine group) was cured. Two out three patients infected with *L. braziliensis* were treatment failures (one in each treatment group).

Miltefosine was effective in curing approximately 70% of the patients compared to 60% in the Glucantime® group (Table 42); the cure rates were similar in children and adults. There was no difference between treatment arms regarding gender, age, duration of illness, and number of lesions. There were 1 to 5 lesions per subject and 45% of subjects had a single lesion. Lesion area of ulceration ranged from 0.8 mm^2 to 1060 mm^2 (mean of 209 mm^2); the mean of surrounding induration per lesion was 325 mm^2.

Table 41: Study Z020a - Diagnostic parasitology – adolescent/adult subjects

Adolescents/Adults

	Miltefosine n=40	Glucantime n=20
Samples Taken by		
Not Available, n(%)	1 (2.5)	
Scraping or Biopsy	39 (97.5)	20 (100.0)
Microscopy		
Not Done, n (%)		1 (5.0)
Positive, n (%)	40 (100.0)	19 (95.0)
Species		
L.braziliensis	1[a] (2.5)	1[b] (5.0)
L.guyanensis	39 (97.5)	19 (95.0)

[a] Subject ID 46.
[b] Subject ID 83.

Pediatrics

	Miltefosine n=20	Glucantime n=10
Samples Taken by		
Scraping, aspiration, or Biopsy	12 (100.0)	10 (100.0)
Microscopy		
Not Done, n (%)	2 (10.0)	
Positive, n (%)	18 (90.0)	10 (100.0)
Species		
L. lainsoni	1[a] (5.0)	
L.braziliensis		1[b] (10.0)
L.guyanensis	19 (95.0)	9 (90.0)

[a] Subject ID 16.
[b] Subject ID 5.

Adolescent/Adults and Pediatrics combined

	Miltefosine n=60	Glucantime n=30
Samples Taken by		
Not Available, n (%)	1 (2)	
Scraping or Biopsy	59 (98)	30 (100)
Microscopy		
Not Done, n (%)	2 (3.3)	1 (3.3)
Positive, n (%)	58 (96.7)	29 (96.7)
Species by PCR		
L.braziliensis	1[a] (1.7)	2[b] (6.7)
L. lainsoni	1[c] (1.7)	
L.guyanensis	58 (96.7)	30 (100.0)

[a] Subject ID 46.
[b] Subject IDs 5 and 83.
[c] Subject ID 16.

Table 42: Study Z020a – Cure rates in adolescent/adult and pediatric subjects

Adolescents/Adults

	Miltefosine n=40	Glucantime n=20	p-value[a]
Cure Rate			
Definitive Cure, n (%)	27 (67.5)	12 (60.0)	.579
Failure, n (%)	13 (32.5)	8 (40.0)	
Reason for Failure			
Lack of initial cure, n	7	4	
Relapse on visit 4 months after EOT, n	2	2	
Relapse on visit 6 months after EOT, n	0	1	
Left study before visit 6 months after EOT, n	4	1	

[a] Fisher exact test. EOT=End of Treatment

Pediatrics

	Miltefosine n=20	Glucantime n=10	p-value[a]
Cure Rate			
Definitive Cure, n (%)	14 (70.0)	6 (60.0)	.690
Failure, n (%)	6 (30.0)	4 (40.0)	
Reason for Failure			
Lack of initial cure, n	4	2	
Relapse on visit 4 months after EOT, n	0	1	
Left study before visit 6 months after EOT, n	2	1	

[a] Fisher exact test. EOT=End of Treatment.

Adolescent/Adults and Pediatrics combined

	Miltefosine n=60	Glucantime n=30	p-value[a]
Cure Rate			
Definitive Cure, n (%)	41 (68.3)	18 (60.0)	.485
Failure, n (%)	19 (31.7)	12 (40.0)	
Reason for Failure			
Lack of initial cure, n	11	6	
Relapse on visit 4 months after EOT, n	2	3	
Relapse on visit 6 months after EOT, n	0	1	
Left study before visit 6 months after EOT, n	6	2	
Cure Rate of evaluable (exclude subjects whose fail reason was left study before visit 6 months)			
Definitive Cure, n (%)	41 (75.9)	18 (64.3)	.306
Failure, n (%)	13 (24.1)	10 (35.7)	

[a] Fisher exact test. EOT=End of Treatment

Comments:

- *Overall the results show miltefosine was effective in the treatment of cutaneous leishmaniasis in Manaus, Brazil, known to be endemic for L. guyanensis.*
- *Speciation was performed by PCR. The details of the assay summarized by the applicant in the NDA submission are same as those in the publication and were insufficient for review of*

the details of the method and its performance characteristics; additionally, the publication cross-references another publication (Garcia et al., 2004[57]) for the PCR assay that reports testing of 34 biopsy specimens from Bolivian patients in a laboratory in Belgium or Peru. However, the laboratory where testing of clinical trial isolates was performed was in Manaus, Brazil and is different from that reported by Garcia et al. (2004)[57]. The details of the method and performance characteristics of the assay were insufficient for review. The applicant states that this was an academic exercise and does not meet the standard of FDA regulations.

5.3.3. Study Z020b

This study was a randomized, open-label, active comparator trial to evaluate the efficacy and safety of oral miltefosine (60 subjects) versus standard therapy with intravenous pentavalent antimony (30 subjects) in the treatment of cutaneous leishmaniasis in Bahia State, Brazil (Study report dated August 7, 2012; Machado *et al*., 2011[59]), known to be endemic for *L. (L.) braziliensis* and *L. (L.) amazonensis*.

Study design

The clinical protocol was same as that summarized above for study Z020a except that the criteria used for the diagnosis of cutaneous leishmaniasis were the presence of a typical ulcerated lesion and a positive intradermal skin test (Montenegro test); the applicant states that the study was performed without amastigote visualization. Subjects were evaluated for clinical response and laboratory parameters (Table 43).

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[59] Machado PR, Ampuero J, Guimarães LH, Villas Boas L, Rocha AT, Schriefer A, Sousa RS, Talhari A, Penna G, and Carvalho EM. Miltefosine in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil: a randomized and controlled trial. PLoS Negl Trop Dis. (2010) 4 (12): e912.

Table 43: Study Z020b - Procedures and schedule

Visit Number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Study Day	Scrn [a]	Day 1	Day 7	Day 14	Day 21	Day 28 or end of treatment	2 weeks after end of treatment	1 month after end of treatment	2 months after end of treatment	4 months after end of treatment	6 months after end of treatment
Assessment											
Informed consent	x										
Medical history	x										
Leishmaniasis history	x										
Parasitology (fecal/blood)[b]	x										
Pregnancy test[c]	x			x				x			
HIV test	x										
Parasitology for *Leishmania*[d]	x										
Montenegro skin test for *Leishmania*	x										
CL Lesion size/ infiltration area	x		x	x	x	x	X	X	X	X	X
Photography of all lesions	x					x	X	X	X		X
Laboratory											
-Hematology[e]	x		x	x	x	x		x			
- Biochemistry[f]	x		x	x	x	x		x			
- Urinalysis[g]	x					x		x			
ECG (Glucantime group)	x					x					
Vital signs[h]	x		x	x	x	x		x	x	x	x
AEs – spontaneous reports		x	x	x	x	x	x	x	x	x	x
Elicited AEs[i]		Daily during treatment									

[a] Within 7 days before 1st dosage (except for parasitological tests of the lesions, that will be valid within the last 4 weeks).
[b] Parasitology for malaria and fecal parasites.
[c] For females of childbearing potential. The start of treatment relative to the date of the pregnancy test was based on the menstrual cycle. If the test was performed within the first half of the menstrual cycle (≤ day 10 of the cycle), treatment was initiated without a waiting period. If the test was performed within the second half of the menstrual cycle (> 10 days), then a two week waiting period during which appropriate contraceptive measures were used was instituted after which a confirmatory pregancy test was performed before start of treatment.
[d] PCR
[e] White blood cells (WBC), hematocrit, platelets.
[f] AST, ALT, alkaline phosphatase, creatinine, BUN, sodium, and potassium.
[g] Blood and protein only.
[h] Temperature, blood pressure, pulse.
[i] Elicited AEs included diarrhea and vomiting.

The criteria used for the diagnosis of cutaneous leishmaniasis were the presence of a typical ulcerated lesion and a positive Montenegro intradermal skin test. Culture and PCR test of lesion specimens was performed for speciation. Testing was performed in the parasitology laboratory of Dr. Albert Schriefer and Rosana S. Sousa.

Montenegro skin test
A Montenegro test (test for cellular immunity to *Leishmania* antigens was performed by the method of Nogueira *et al.* (2008)[60]. The *Leishmania* antigen (25 µg in 0.1 mL) used for intradermal skin testing, on the left forearm, was obtained from *L. amazonensis* strain (MHOM-BR-86BA-125). The largest diameter of induration was measured at 48-72 hours. The test was considered positive if induration was greater than 5 mm. The data supporting such a threshold were not included in the submission or in the publication.

[60] Nogueira MF, Goto H, Sotto MN, and Cucé LC. Cytokine profile in Montenegro skin test of patients with localized cutaneous and mucocutaneous leishmaniasis. Rev Inst Med Trop Sao Paulo (2008) 50:333-337.

Leishmania parasitology
Confirmation of the presence of *Leishmania* parasites was performed on specimens obtained by aspiration or biopsy. The presence of *Leishmania* was examined by a positive culture or PCR, not by microscopy as originally stated in the protocol.

To culture *Leishmania*, a needle aspiration of a skin lesion was collected and aspirates were cultured in Nicolle-McNeal-Novy medium overlaid with modified liver infusion triptase medium. Cultures were incubated at 25°C and examined twice weekly for parasites. To diagnose for the presence of *Leishmania* and also to identify *Leishmania* species by PCR, DNA isolation was carried out from biopsy samples using the Wizard Genomic DNA purification kit (Promega Corporation, USA). *Leishmania* genus detection utilized the primers 59-(G/C)(G/C)(C/G)CC (A/C)CTAT(A/T)TTA CAC CAA CCC C-39 and 59-GGG GAG GGG CGT-39. Detection of the subgenus *Viannia* utilized the primers 59-GGGGTTGGTGTAATATAGTGG-39 and 59-CTAATTGTGCACG-39. The *Leishmania* specific band consisted of 120 base pairs and that for *Viannia* of 750 base pairs. The applicant states that PCR as performed identified the genus (*Leishmania)* and the subgenus (*Viannia)* but not the species. In Bahia, human cutaneous leishmaniasis is known to be caused by *L. (V.) braziliensis* and *L. (L) amazonensis* (Grimaldi et al-1989[61]). The authors state that determining that the *Viannia* subgenus was present was equivalent to determining that *L. (V.) braziliensis* and not the other possibility, *L. (L.) amazonensis*, as the infecting parasite.

No parasitological measurements were performed during or post-treatment.

Results
There were 1-3 lesions per subject. The majority of subjects had a single lesion (80%). Lesion area of ulceration ranged from 11.8 mm^2 to 1519.7 mm^2, with a mean of 419 mm^2. There was a mean of 244 mm^2 of surrounding induration per lesion. For no parameter did the values differ significantly ($p > 0.05$) between the groups.

Montenegro skin test
The applicant states that although the results of the skin test were not provided on the study CRFs, the principal investigator certified that all subjects were positive by this assay.

Parasitology by culture or PCR
In the two treatment groups, 77-80% of adolescent/adult subjects and 50 – 70% of pediatric subjects were parasitologically-positive by either culture or PCR (Table 44). *L. (V.) braziliensis*, based on typing for the subgenus by PCR, identified in 41 biopsy samples by PCR, was the only species found in the present study.

[61] Grimaldi G Jr, Tesh RB, and McMahon-Pratt D. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. Am J Trop Med Hyg. (1989) 41:687-725.

Table 44: Study Z020b - Diagnostic parasitology

Adolescent/Adult Subjects

	Miltefosine N=40	Glucantime N=20
Samples Taken by		
Biopsy or aspiration	40 (100.0)	20 (100.0)
Culture		
Not Done, n (%)	5 (12.5)	2 (10.0)
Negative, n (%)	10 (25.0)	5 (25.0)
Positive, n (%)	25 (62.5)	13 (65.0)
PCR		
Negative, n (%)	14 (35.0)	6 (30.0)
Positive, n (%)	26 (65.0)	14 (70.0)
Parasitology Test Either Culture OR PCR postive		
Negative, n(%)	9 (22.5)	4 (20.0)
Positive, n(%)	31 (77.5)	16 (80.0)

Pediatric Subjects

	Miltefosine N=20	Glucantime N=10
Samples Taken by		
Biopsy or aspiration	20 (100.0)	10 (100.0)
Culture		
Not Done, n (%)	2 (10.0)	
Negative, n (%)	10 (50.0)	4 (40.0)
Positive, n (%)	8 (40.0)	6 (60.0)
PCR		
Not Done, n (%)	4 (20.0)	3 (30.0)
Negative, n (%)	10 (50.0)	3 (30.0)
Positive, n (%)	6 (30.0)	4 (40.0)
Parasitology Test Either Culture OR PCR positive		
Negative, n(%)	10 (50.0)	3 (30.0)
Positive, n(%)	10 (50.0)	7 (70.0)

All Subjects

	Miltefosine N=60	Glucantime N=30
Samples Taken by		
Aspiration or biopsy	60 (100.0)	30 (100.0)
Culture		
Not Done, n (%)	7 (11.7)	2 (6.7)
Negative, n (%)	20 (33.3)	9 (30.0)
Positive, n (%)	33 (55.0)	19 (63.3)
PCR		
Not Done, n (%)	4 (6.7)	3 (10.0)
Negative, n (%)	24 (40.0)	9 (30.0)
Positive, n (%)	32 (53.3)	18 (60.0)
Parasitology Test Either Culture OR PCR postive		
Negative, n(%)	19 (31.7)	7 (23.3)
Positive, n(%)	41 (68.3)	23 (76.7)

In adolescent/adult subjects the cure rates were higher in patients treated with miltefosine compared to Glucantime treated group (Table 45); however, in pediatric patients the cure rates were lower in the miltefosine group compared to Glucantime treated group. Overall, definitive cure rates were higher in subjects treated with miltefosine compared to Glucantime.

Table 45: Study Z020b - Cure rates and reasons for failure

Adolescent/Adult Subjects

	Miltefosine N=40	Glucantime N=20	p-value[a]
Definitive Cure Rate			
Definitive Cure, n (%)	34 (85.0)	9 (45.0)	.002
Failure, n (%)	6 (15.0)	11 (55.0)	
Reason for Failure			
Lack of initial cure, n	4	6	
Relapse on visit 4 months after EOT, n	1	1	
Left study before visit 6 months after EOT, n	1	4	
Cure Rate of evaluable (exclude subjects whose fail reason was left study before visit 6 months)			
Definitive Cure, n (%)	34 (87.2)	9 (56.3)	.027
Failure, n (%)	5 (12.8)	7 (43.8)	

[a]Fisher's Exact test. EOT=End of Treatment

Pediatric Subjects

	Miltefosine N=20	Glucantime N=10	p-value[a]
Definitive Cure Rate			
Definitive Cure, n (%)	13 (65.0)	9 (90.0)	.210
Failure, n (%)	7 (35.0)	1 (10.0)	
Reason for Failure			
Lack of initial cure, n	4	0	
Relapse on visit 4 months after EOT, n	1	0	
Left study before visit 6 months after EOT, n	2	1	

[a]Fisher's Exact test. EOT=End of Treatment

All Subjects

	Miltefosine N=60	Glucantime N=30	p-value[a]
Definitive Cure Rate			
Definitive Cure, n (%)	47 (78.3)	18 (60.0)	.083
Failure, n (%)	13 (21.7)	12 (40.0)	
Reason for Failure			
Lack of initial cure, n	8	6	
Relapse on visit 4 months after EOT, n	2	1	
Left study before visit 6 months after EOT, n	3	5	

[a]Fisher's Exact test. EOT=End of Treatment

	Miltefosine N=60	Glucantime N=30
Cure Rates with Positive Parasitology Test		
Definitive cure, n(%)	33 (80.5)	12 (52.2)
Failure, n(%)	8 (19.5)	11 (47.8)
Cure Rates with Negative Parasitology Test		
Definitive cure, n(%)	14 (73.7)	6 (85.7)
Failure, n(%)	5 (26.3)	1 (14.3)

Although the number of patients that were parasitologically negative was small, the cure rates were similar in parasite-negative and parasite-positive subjects (Table 45). All the subjects were Montenegro skin-test positive in response to *Leishmania* antigens.

Comments:

- *Overall, the results show miltefosine was effective in the treatment of cutaneous leishmaniasis in Bahia, Brazil known to be endemic for L. braziliensis and L. amazonensis.*
- *Samples were obtained by biopsy or aspiration. To identify Leishmania parasites, the aspirates were cultured or the biopsy was subjected to PCR. PCR as performed identified the genus (Leishmania) and the subgenus (Viannia) but not the species. Therefore the Leishmania species was presumed to be L. (V.) braziliensis. In Bahia, cutaneous leishmaniasis is known to be caused by L. (V.) braziliensis and L. (M.) amazonensis. The applicant states that this was an academic study for which final clinical study report was retrospectively written and documentation of assay sensitivity/specificity/etc. according to US FDA guidelines were not available for the PCR method.*
- *For the patients who were negative by culture or by PCR, the diagnosis of leishmaniasis was provided by a positive Montenegro Test [positive response to Leishmania antigen (L. (L.) amazonensis) injected into the skin]. The Montenegro Test, performed and interpreted as is the Tuberculin Test, is a standard diagnostic technique throughout Leishmania-endemic regions, although documentation of assay sensitivity/specificity according to US FDA guidelines is not available. It is unclear if the positive skin test findings are due to common antigens between the Leishmania species known to be prevalent in Bahia and L. amazonensis antigens used for the antigen test.*
- *Post treatment parasitological observations were not performed.*

5.3.4. Study Soto

This study was a randomized, open-label, active comparator trial to evaluate the efficacy and tolerance of oral miltefosine (n=54) with Glucantime® (n=26) for the treatment of cutaneous leishmaniasis in Bolivia (Study report dated July 17, 2012 and Soto *et al*., 2008[62]) known to be endemic for *L. braziliensis*. Miltefosine was administered at a target dose of 2.5 mg/kg/day orally for 28 days. Glucantime® was administered at a dose of 20 mg/kg/day intramuscularly.

The primary efficacy endpoint was clinical cure which was defined as complete re-epithelialization of all lesion ulcers at 6 months after the end of therapy.

Study design

Inclusion criteria

- Age ≥ 12 years.
- Gender: male; and female if not pregnant or lactating, and if contraceptive measures were agreed to be used during the time of the treatment and for 2 months after the last dose of the study drug.
- History of leishmaniasis: Ulcerative lesion suggestive of cutaneous leishmaniasis.
- Parasitology: *Leishmania* seen in histopathological examination of lesion material.

[62] Soto J, Rea J, Balderrama M, Toledo J, Soto P, Valda L, and Berman JD. Short Report: Efficacy of miltefosine for Bolivian cutaneous leishmaniasis. Am J Trop Med Hyg (2008) 78 (2): 210-211.

- History of treatment for leishmaniasis: No previous treatment for leishmaniasis, or if previously treated, the treatment must before have been at least 2 months prior.
- Informed consent should have been obtained.

Exclusion criteria

- Concomitant diseases: no clinically significant abnormalities on physical examination.
- Laboratory values: without clinically significant abnormalities in hematology, transaminase, or creatinine.
- Unable to comply with protocol: inability to complete the requirements of the study and to attend the follow up visits.

Anti-leishmanial agents were forbidden for 2 months prior to the first administration of drug, and for the duration of the study.

Efficacy was evaluated by measuring the size of lesions at the beginning of therapy, the end of therapy, and at 1, 3, and 6 months after the end of therapy (Table 46).

Table 46: Study Soto - Assessments schedule

Event	Day 0	Treatment	End of Treatment	1 month[a]	3 months[a]	6 months[a]
Consent	X					
History	X		X	X	X	X
Physical exam	X		X	X	X	X
Parasitology	X		X	X	X	X
Clinical Laboratory[b]	X		X	X	X	X
Study Treatments		Miltefosine: Days 1-to-28 Glucantime: Days 1-to-20				
Adverse Events		Daily during treatment				

[a] If medically needed.
[b] Hemoglobin, white blood cell count, platelet count, serum glutamic oxaloacetic transaminase (SGOT) creatinine.

For a subject to be clinically cured, all lesions had to cured based on complete re-epithelialization at month 6 (Table 47).

Table 47: Study Soto - Efficacy response definitions

Endpoint	Definition
Cure	100% re-epithelializaton of all initial lesions
Failure	Increase in lesion size at the end of therapy or at 1 month after therapy by 50% in comparison with the initial measurements, less than 50% decrease in size at 3 months after therapy in comparison with the initial measurements, lack of 100% decrease in size at 6 months after therapy; relapse; appearance of new lesions
Lost to Follow-up	Did not come to final study evaluation

A direct examination of cutaneous leishmaniasis lesions was also performed at each visit with lesions photographs, biopsies, and parasitological cultures performed when considered necessary. Parasitological examinations were performed by microscopic examination of lesion smears or biopsies. Cultures and species identification were not performed.

Results

Clinical cure, at 6 months after the end of therapy, in patients treated with miltefosine were similar to those treated with Glucantime (Table 48).

Table 48: Study Soto - Clinical response rates – mitt analysis set

	Miltefosine N= 40		Glucantime N=18		p-value[a]
	n	%	n	%	
Cure	32	80.0%	13	72.2%	0.83
95% CI		64.3%, 90.9%		49.1%, 87.5%	
Failure	6	15.0%	1	5.5%	
Lost to follow-up	2	5.0%	4	22.2%	

[a] Fisher's Exact Test

Comments:

- *Clinical cure rates were similar in the miltefosine and Glucantime treatment groups in Bolivian patients with cutaneous leishmaniasis. Based on epidemiological studies L. braziliensis is prevalent in this area.*
- *Differences in the cure rates against presumed L. braziliensis in Bolivia with that in Guatemala (Study 3168) suggests that efficacy in one endemic region cannot be relied on to pertain to other endemic regions, even if the apparently "same" species of Leishmania is prevalent in both regions. A decrease in response rate due to decreased sensitivity to miltefosine cannot be ruled out.*
- *Post treatment parasitology was not performed.*

5.3.5. Study 3092

This was an open-label, single center trial to assess efficacy and safety of 3 sequential dosage groups of oral miltefosine, with dosing for 20 days, in patients with South American cutaneous leishmaniasis (Study No. D-18506/1300-3092-01; Soto *et al*., 2001[63]); from the publication it appears that the study was conducted in male Colombian soldiers with cutaneous leishmaniasis; Based on epidemiological findings, *L. panamensis, L. braziliensis, L chagasi, L. guyanensis,* and *L. amazonensis* are prevalent in Colombia. The protocol was amended to include a 4th dose group with dosing for 28 days.

[63] Soto J, Toledo J. Gutierrez P, Nicholls J, Engel J, Fischer C, Voss A, and Berman J. Treatment of American cutaneous leishmaniasis with miltefosine, an oral agent. Clin Infect Dis (2001) 33:e57 -61.

Study design

Inclusion criteria

- Written informed consent.
- Male aged 16-60 years.
- Newly diagnosed or resistant/relapsing cutaneous leishmaniasis. Confirmation by aspirate and parasitological examination of each lesion.
- Typical cutaneous ulceration.
- In case of prior therapy: end of prior therapy longer than 4 weeks ago and cutaneous lesion(s) must be equal or worse than at the end of the prior therapy.
  (criterion added as *per-protocol amendment 2).*
- Karnofsky performance status 80% or higher.
  (as per amendment 1; *initial protocol: 30% or higher)*
- Thrombocyte count: ≥ 120 x 10^9/L
  (as per amendment 1; *initial protocol:* ≥ 50 x 10^9*L or higher)*
- Leukocyte count: ≥ 4 x 10^9/L
  (as per amendment 1; *initial protocol:* ≥ 2 x 10^9*/L or higher)*
- Hemoglobin: ≥ 10 g/100 mL.
  (as per amendment 1; *initial protocol:* ≥ 6 *g/100 mL or higher)*
- ASAT, ALAT, AP; serum bilirubin; prothrombin time, serum creatinine: Within normal limits.
  (as per amendment 1; *initial protocol required:*
  ASA T, ALA T, AP; serum bilirubin: < 3 *times upper limit of normal range; prothrombin time:* < 5 *seconds above control; serum creatinine: < 180 µmol (< 2.0 mg/100 mL).*

Exclusion criteria

- Major surgery within last 2 weeks.
- Concomitant treatment with any other drug(s) except antibiotics, antimalarial medication, iron/folate substitution and NSAIDs.
- Any non-compensated or uncontrolled condition, such as congestive heart failure, active tuberculosis, malignant disease or severe malaria.
- Pre-existing impaired vision.
- Any condition which compromises ability to comply with the study procedures.
- Inadequate contraception in men.
- Life expectancy less than 4 weeks *(criterion deleted* as *per amendment 1)*

Patients were administered the following study medication:
• Group 1: 50 mg/day on day 1-20.
• Group 2: 50 mg/day on day 1-7, followed by 100 mg/day on day 8-20.
• Group 3: 100 mg/day on day 1-7, followed by 150 mg/day on day 8-20.
• Group 4: 150 mg/day on day 1-28.

Definite cure required full epithelialization of skin ulceration and/or disappearance of inflammatory induration of the skin at the end of the 6-month follow up period.

Assessments of efficacy

Subjects were evaluated for clinical response at different time intervals up to 6 months after end of therapy (Table 49). Parasitological measurements were performed if clinically indicated.

Each lesion was measured in size and grade of epithelialization. A photograph was to be taken according to the approved procedure at the site of the investigator. Lesions were either ulcerations or inflammatory indurations of the skin.

- *Parasitological measurements:*

From each lesion that was not epithelialized an aspirate was collected. The aspirates were cultured for promastigotes and stained with Giemsa. Lesions were also superficially scraped for culture and staining.

Proof of infection included the demonstration of motile promastigotes cultured from lesions or microscopic identification of *Leishmania* amastigotes in the direct stain.

Table 49: Study 3092 – Schedule and procedures for dose group 4 (protocol amendment 3) Group 1-3: "Day 29" assessments on Day 21; "Day 42" assessment on Day 34

Item / Assessment	Screen	Assessment during treatment				End of treat-ment	Follow up #2)		Final evaluation #3)
	within 7 days before 1st dose	Day 1	Day 8	Day 15, 21	Day 29	#1) (if not Day 29)	Day 42	Month 3	Month 6
Informed consent	X								
Registration	X								
Medical history	X								X
Medication (distribution)		X							
Efficacy									
- Parasitology #6)	X							X #6)	X #6)
- Clinical evaluation + photo documentation	X				X	X	X	X	X
Clinical assessment & adverse events #4a)	X	X	X	X	X	X #4b)	X #4c)	X	X
- Ophthalmology	X		X	X	X	X	X	X	X
Laboratory #5)									
- Hemogram	X		X	X	X	X		X	X
- Chemistry	X		X	X	X	X		X	X
- Urinalysis	X		X	X	X	X		X	X
Others #5)									
- Parasitology (stool) (malaria)	X X								

#1) A full assessment will be required whenever treatment is discontinued. The reason(s) for discontinuation will be documented. This will also apply to patients withdrawing from the study for whatever reason.

#2) A follow up assessment will be performed two weeks after the last administration of the study medication (= day 42; *day 34 for group 1-3*). All patients who had no clinical failure on day 42 (*day 34 for group 1-3)* will also have a follow up assessment 3 months after end of treatment (= month 3).

#3) The final evaluation is scheduled 6 months after end of treatment. In a patient with a relapse, the final evaluation will be performed whenever the relapse (= clinical failure) is diagnosed.

#4a) For expedited notification of "Serious Adverse Events" (SAE) see chapter 11.5.

#4b) AEs persisting at the end of treatment for which a causal relationship to the study medication cannot be ruled out, will be followed up to assess reversibility. Intervals will be adapted according to kind and intensity but will not exceed 4 weeks (please see also chapter 9.4.).

#4c) Follow up of adverse events: After the day 42 (*day 34 for group 1-3)* assessment a reassessment of all AEs which existed / occurred since the visit on day 42 (*day 34 for group 1-3*), will be done 4 weeks after end of treatment (=post-treatment follow up).

#5) Additional examinations will be performed as medically indicated.

#6) After start of study treatment parasitology will only be performed if a skin lesion has not changed or increased in size.

Clinical response was defined as follows:

- **Definite cure:** 100% epithelialization of all ulcers at the 6 months follow up examination.
- **Apparent cure:** 100% epithelialization of all ulcers 2 weeks after end of treatment.
- **Initial partial cure:** 75 - 99% epithelialization and no increase in size of lesions 2 weeks after end of treatment.

- **Clinical failure:** appearance of any new lesions or enlargement of previously documented lesions or less than 75% epithelialization of lesions.

Parasitological response was defined as follows:

- **Parasitological cure:** stain and culture negative for parasites.
- **Parasitological failure:** presence of parasites in stain or culture.

No parasitology was to be performed after lesions were epithelialized, and definition of cure was based on clinical criteria only.

If a patient who was not clinically cured at 2 months after end of treatment, rescue treatment with parenteral standard treatment was administered. Such a patient was clinical failure at any time after end of study treatment.

Results

There were 20 subjects in the ITT and 18 in the PP (fulfilled the selection criteria and were administered the scheduled trial medication on at least 90% of the planned treatment days and were assessed at least for apparent cure) population treated with miltefosine at the dose and duration (Group 4) the applicant intends to recommend in the labeling; 4 patients had previous treatment failure. Patients who dropped out early due to lack of efficacy were not excluded from the PP population. The results showed cure rates at two weeks and 6 months after end of treatment were about 80% in Group 4 in the ITT and PP population. There was no relationship between presenting lesion size and cure (Table 50).

No parasitology information was included in the submission by the applicant. However, the authors state in the publication that *Leishmania* species was identified by isoenzyme electrophoresis. *L. panamensis* was identified in 10 patients of whom 3 were in group 4. *L. amazonensis* was identified in 5 patients and none of them were in group 4.

Comments:
Cure rates at two weeks and 6 months after end of treatment were about 80% in Group 4 in the ITT and PP population in subjects from Colombia known to be endemic for L. panamensis, L. braziliensis, L chagasi, L. guyanensis, and L. amazonensis. There was no relationship between presenting lesion size and cure.

Table 50: Study 3092 – Cure rates

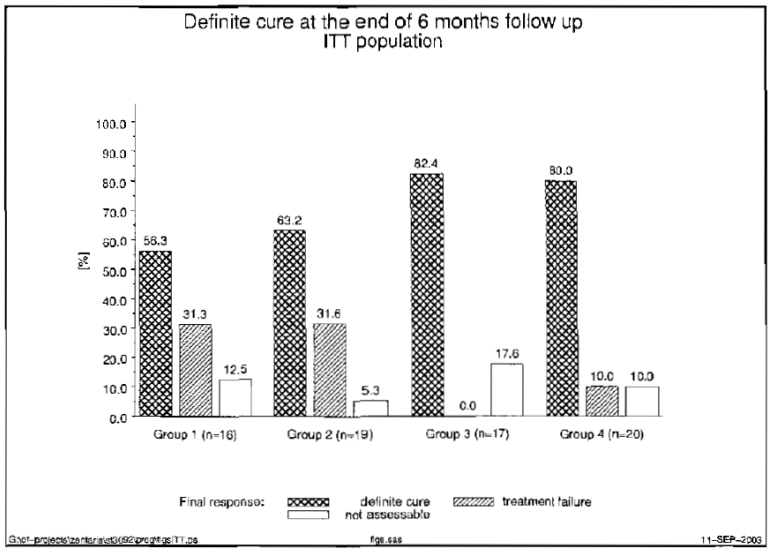
Cure rates at 2 weeks after end of treatment in ITT population

	Group 1 (N=16)		Group 2 (N=19)		Group 3 (N=17)		Group 4 (N=20)	
Cure status	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
apparent cure	8	50.0	7	36.8	13	76.5	16	80.0
partial cure	3	18.8	9	47.4	3	17.6	1	5.0
clinical failure	5	31.3	3	15.8	0	0.0	0	0.0
missing	0	0.0	0	0.0	1	5.9	3	15.0

Cure rates at the end of 6 months follow-up

ITT population	Group 1	Group 2	Group 3	Group 4
Rate of definite cure	9/16 (56.3%)	12/19 (63.2%)	14/17 (82.4%)	16/20 (80.0%)
95% (90%) lower confidence bound	33.3% (37.5%)	41.8% (45.9%)	60.4% (64.8%)	59.9% (63.9%)

PP population	Group 1	Group 2	Group 3	Group 4
Rate of definite cure	9/14 (64.3%)	12/18 (66.7%)	14/14 (100.0%)	16/18 (88.9%)
95% (90%) lower confidence bound	39.0% (43.7%)	44.6% (48.8%)	80.7% (84.8%)	69.0% (73.1%)



Response assessment after 6 months of follow up Proportions [%]

5.3.6. Study Z026

This was a phase 2b study (Report no. D-18506/Z026-01) to evaluate the efficacy of orally administered miltefosine in patients with cutaneous leishmaniasis in Kabul, Afghanistan. Kabul is known to be endemic for *L. tropica.* This was a randomized controlled trial with 3 parallel arms that include oral miltefosine, currently used intra-lesional sodium stibogluconate or intra-muscular administration of sodium stibogluconate. The intramuscular arm was closed prematurely due to poor acceptance.

Study design

Inclusion criteria

- Male and female adult patients.
- Children above 10 years of age.

- Patients presenting with single active lesion; due to slow enrolment, later patients with two active lesions were included as well.
- Parasitological confirmation.

Exclusion criteria

- Lactation, pregnancy (to be determined by adequate test) or inadequate contraception in females of childbearing potential for treatment period plus two months.
- Prior anti-leishmania treatment.
- Any non-compensated or uncontrolled condition, such as active tuberculosis, malignant disease, malaria, HIV, or other major infectious disease.
- Major surgery within the last month.
- Lack of ability or willingness to give informed consent (patient and/or parent/legal representative).
- Anticipated non-availability for study visits or procedures.

Miltefosine was administered orally at a dose of 100-150 mg/day (target dose approximately 2.5 mg/kg/day). Blisters with capsules sufficient for 3-4 days were delivered to patients (bi-weekly visits during treatment phase). Sodium stibogluconate (Pentostam® was administered either

- Intra-lesional (three to five 2-7mL injections, every 4-6 days), or
- Intra-muscular (20 mg/kg bodyweight for 21 days).

Assessment of lesions for cure was performed at weeks 2, 4-5, 8-11, and 12-16 as well as 6 months after end of treatment. Clinical cure was defined as complete re-epithelialization; failure was defined as incomplete re-epithelialization at 2 months follow-up or any relapse within the period of follow-up. Clinical observations included repeat measurements of lesion size.

Parasitological cure was evaluated by microscopy based on a scoring scale (Table 51).

Table 51: Study Z026 – parasitological response scoring scale

Score	Number of amastigotes	per number of fields
0	no amastigotes	per 1000 fields
1+	1-10	per 1000 fields
2+	1-10	per 100 fields
3+	1-10	per 10 fields
4+	1-10	per field
5+	10-100	per field
6+	>100	per field

Results

Of the 344 patients with parasitologically-confirmed cutaneous lesions enrolled, 104, 102, and 48 patients completed treatment with oral miltefosine or intra-lesional and intra-muscular stibogluconate, respectively. The results showed efficacy of oral treatment with miltefosine was comparable to intra-lesional or intra-muscular sodium stibogluconate after 2 to 4 months of follow-up (Table 52); a considerable number of patients were lost to follow-up.

Table 52: Study Z026 – Clinical cure rates*

	Miltefosine	Intramuscular SSG	Intralesional SSG
Number (rate) of patients cured	29/46 (63%)	15/23 (65%)	23/32 (72%)

*Cure rates based on patients that completed 2-4 months of follow-up

Comments:
The efficacy of miltefosine was comparable to sodium stibogluconate in Kabul, Afghanistan known to be endemic for L. tropica.

5.4. Diffuse cutaneous leishmaniasis

5.4.1 Study Z027

This was a phase 2, uncontrolled, single arm pilot trial to evaluate the efficacy and safety of miltefosine (2-2.5 mg/kg/day; duration of treatment varied[64]) for the treatment of diffuse cutaneous leishmaniasis in about 12 to 16 patients in Venezuela (Study report no. D-18506/Z027-01 dated July 2005; Zerpa *et al*., 2007[41]). Based on epidemiologic findings, *L. amazonensis* is the prevalent *Leishmania* species in this region.

Study design

Inclusion criteria:

- Patients with diffuse cutaneous leishmaniasis in whom other therapy schemes had failed.[65]
- Laboratory tests within normal limits including: complete hematology, blood sugar, urea, creatinine, total and partial proteins, electrolytes (Na, K, Cl), liver enzymes (GOT, GPT, ALP), bilirubin, lipids.
- HIV-negative, VDRL- negative, Hepatitis B/ C Ag- negative.
- No pre-existing symptoms that could be misunderstood for adverse reaction (e.g., diarrhea, vomiting).

Exclusion criteria:

- Laboratory test abnormalities.
- Women at fertile age not using contraceptives.
- Renal, hepatic, ophthalmologic and gastrointestinal pathological background.
- Alcohol or drug abuse.
- Patients with previous history of severe allergic reactions.
- History of psychiatric disease.
- Non-localizable address.
- Immunosuppressed patients.

[64] Initial plan was to treat patients for 28 days; after observation of relapses, patients were re-treated until disappearance of lesions (up to 4 months of treatment). Protocol was amended for newly enrolled patients and treatment duration was extended to 6 months

[65] The authors state in the publication that all patients had been followed for years and had received multiple cycles of treatment with anti-*Leishmania* drugs and immunotherapy with killed *Leishmania* promastigotes and Bacille Calmette-Guerin.

Subjects were evaluated for clinical response (regression of lesions) and photographs were taken before treatment, weekly during treatment, at end of treatment, and at 3 and 6 months after end of treatment.

Parasitological assessments:
Skin biopsies were collected for histological sections and smears (stained with hematoxylin-eosin and Giemsa respectively), rabbit blood-agar medium cultures (observed weekly for the presence of promastigotes) for *Leishmania*, inoculation of hamsters in the footpad (observed weekly for the presence of appearance and persistence of infiltrated lesions), and PCR. Intradermal Leishmanin skin test (LST) and parasitic load in biopsy smear slides were also assessed.

Identification of *Leishmania* species was based on isoenzymatic and molecular approaches. The isoenzymatic technique was based on starchgel electrophoresis using 13 isoenzyme systems, according to the method of Rioux *et al*. (1990)[66].The molecular approach was based on the sequencing of both strands of a 567-bp region of the RNA polymerase II large subunit gene using primers RP720-D 5'-AAGTACCAGCAGTCCCTCATC-3' and RPOII-FOR 5'-GCAGCCGCACAATGCGCT-3'. Sequences were compared with a personal data bank of various *Leishmania* species.

Results
All patients had received multiple previous treatment schemes without cure, 4 patients had received more than 30 previous treatment courses of Glucantime. Upon observation of significant improvement of lesions and good tolerability of miltefosine treatment after 28 days, it was decided to prolong treatment up to clearance of lesions; a first cohort of 8 patients received miltefosine treatment for periods ranging from 2.5 to 4 months, leading to both clinical and parasitological clearance of the skin lesions. After a treatment-free interval of 1 to 5 months, however, all patients relapsed and all but one started re-treatment with miltefosine.

Subsequently recruited new patients were treated with miltefosine according to a modified protocol, allowing initial treatment courses of 6 months.

The applicant included results of 12 subjects whereas results for 16 subjects were available in the publication. The disease duration ranged from 1.5 to 30 years and had received multiple cycles of prior treatment with pentavalent antimonials, immunotherapy and, in two cases, amphotericin B, with limited improvement and subsequent relapse. *L. amazonensis* was identified in cultures of specimens from 11 subjects, *L. mexicana* in two, and 3 were not definitively identified (Table 53). The leishmanin reaction was negative in all of the patients and the histological sections showed a vacuolated macrophagic granuloma invaded by numerous lymphoid and plasma cells, with numerous *Leishmania* amastigotes in the macrophages.

A gradual increase in clinical improvement was reported and by day 75, 10 patients showed 100% improvement and in 5 subjects about 80–95% improvement (Table 53). By day 30 very few parasites in the granulomas were observed. The granulomas disappeared in the majority of the patients by day 75 and the only observation was nonspecific perivascular inflammatory infiltration.

[66] Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, and Perieres J. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Ann Parasitol Hum Comp (1990) 65:111–25.

In skin smears, parasites were observed until day 30 in 6 patients, until day 45 in 7 and until day 60 in 2. One remained positive until day 190, when treatment was suspended in the absence of a detectable therapeutic response.

Growth of parasites in culture medium as well as by inoculation in hamsters was observed in all the patients before treatment. A majority of the patients (15/16) were smear and/or biopsy negative by Day 63. Inoculation in hamsters remained positive until day 45 and was negative by Day 75 in 14 of the 16 subjects (Table 53).

Twelve patients relapsed after the first treatment cycle, including those who received 6 months of treatment. A second cycle was administered and five did not respond, with persisting clinical lesions of diffuse cutaneous leishmaniasis and presence of parasites in skin smears.

The reduction in the parasitic burden was related to clinical improvement beginning on day 15 in skin smears and tissue sections. Nevertheless, the more sensitive method of animal inoculation showed the persistence of parasites until day 120 in a patient who showed clinical improvement of 100% at day 75 and disappearance of parasites in smears and histological sections at day 45 suggesting relapse.

In biopsies obtained from these relapsed patients, the presence of granulomas formed by vacuolated macrophages containing numerous parasites was reported. Therefore, the second group received additional treatment for 6 months. These patients also presented new lesions 30 days after finishing treatment.

The leishmanin reaction remained negative in 15 patients after treatment was terminated; a single patient who had not relapsed had a leishmanin reaction of 13 mm at 1 year after treatment was completed suggesting that the use of miltefosine and reduction in the parasite burden was not accompanied by an alteration in the immunological response of the host to *Leishmania*. Absence of an appropriate immune response to the parasite may make them vulnerable for relapse.

Comments:
Overall, the results suggest that miltefosine treatment was effective in the treatment of patients with severely affected diffuse cutaneous leishmaniasis in Venezuela , a region known to be endemic for L. amazonensis and L. mexicana. While complete healing and negative parasitology of skin lesions was achieved after treatment durations ranging from 8- 12 weeks, relapses were noted in nearly all patients suggesting development of resistance to miltefosine or inability to restore immune responses.

Table 53: Study Z027 - Miltefosine in the treatment of diffuse cutaneous leishmaniasis, first cycle of treatment

Patient	Age (years)	Weight (kg)	Daily dose (mg)	Day of suspension of treatment	Molecular and isoenzymatic identification of *Leishmania*, MON
1	32	60	150	120	*L. amazonensis*, MON – 41
2	15	49	150	75	*L. amazonensis*, MON – 41
3	45	66	150	101	*L. amazonensis*, MON – 41
4	20	62	150	70	*L. amazonensis*, MON – 41
5	19	75	150	75	*L. amazonensis*, MON – 41
6	33	59	150	111	*L. amazonensis*, MON – 41
7	14	30	100/50	84	*L. mexicana*, MON – 40
8	48	65	100	111	*L. amazonensis* (contaminated culture of the strain)
9	51	70	150	218	*L. mexicana*, MON – 40
10	22	70	150	190	*L. amazonensis*, MON – 41
11	38	58	150	190	*L. amazonensis* (contaminated culture of the strain)
12	40	80	150	190	*L. amazonensis*, MON – 41
13	33	51	150	140	*L. amazonensis*, MON – 41
14	10	30	50 × 40 days; 50/100[a]	190	*L. amazonensis*, MON – 41
15	3	19	50	114	*L. mexicana* (contaminated culture of the strain)
16	8	20	50	73	*L. amazonensis*, MON – 41

[a]Patient received 50 mg daily for 40 days, then 50 or 100 mg on alternating days.

Patient	100% clinical improvement (day)	Parasites detected in smears and biopsies (day)	Positive inoculation in hamsters (day)	Day of relapse after treatment
1	75	45	120	140
2	60	45	60	87
3	75	30	75	156
4	60	30	45	70
5	75	45	60	83
6	60	45	60	64[a]
7	75	60	60	30
8	90	45	60	95
9	75	45	45	156
10	105	60	45	70
11	105	30	61	No, 1 year
12	90	60	45	No response, 14[b]
13	85	45	30	Patient lost
14	Only 50% improvement	190	190	No improvement
15	65	63	15	114[c]
16	57	43	15	30

[a]New lesions appeared on day 64 of the second cycle of treatment.
[b]Did not respond to a second cycle of treatment.
[c]New lesions during treatment, first cycle.

6. THE LABELING

6.1. Applicant's version of the microbiology section of the labeling

12.1 Mechanism of Action
Miltefosine is an antileishmanial agent. *[see Microbiology (12.4)]*

12.4 Microbiology
Mechanism of Action
The specific mode of action of miltefosine (b) (4) against *Leishmania* species is unknown. The mechanism of action of miltefosine is likely to involve interaction with lipids (phospholipids and sterols), including membrane lipids.

Activity *In Vitro* and *In Vivo*
Miltefosine has (b) (4) anti-leishmanial activity *in vitro* and in (b) (4)

Drug Resistance
(b) (4)

6.2. Comments

- *Under the subheading 'Mechanism of action', the applicant has proposed to state that* (b) (4) *While this is appropriate, the studies also show that miltefosine inhibits cytochrome c oxidase (mitochondrial function) and causes apoptosis-like cell death; such information should be included in the labeling.*
- *Under the subheading 'Activity in vitro and in vivo', the applicant has proposed to state that "Miltefosine has direct anti-leishmanial activity in vitro and in* (b) (4) *Based on the current Division practice, it is recommended that activity in* (b) (4) *should not be included and should be replaced with clinical infections. Additionally, a reference to Clinical Studies, Section 14 should be made and the following sentence should be added under the subheading Activity in vitro and in vivo':*
  Sensitivity of different Leishmania species as well as different strains of a Leishmania species to miltefosine may vary in different geographic regions.
- *Under the subheading 'Drug resistance' the applicant has proposed to add* (b) (4) *Studies reviewed showed that some strains of L. braziliensis with intrinsic resistance to miltefosine have been identified. Additionally, drug resistance could be due to a decrease in miltefosine accumulation which is thought to be due to either an increase in drug efflux, mediated by the overexpression of the ABC transporter P-glycoprotein and/or a decrease in drug uptake by the inactivation of the miltefosine transport machinery that consists of the miltefosine transporter and its beta subunit. Mutation in the transporter gene was reported in a relapsed patient in one study. It will be worthwhile adding this information to the labeling.*
- *The applicant proposes to state the following in the 'Highlight' and 'Indication' section that*
  - *Visceral leishmaniasis is due to Leishmania donovani.*
  - *Cutaneous leishmaniasis is due to Leishmania (L) viannia (v) subgenus (L. v. braziliensis, L.v. guyanenesis, L.v. panamensis).*
  - *Mucosal leishmaniasis is due to L. v. braziliensis, L. v. guyanenesis, and L.v. panamensis.*

The different Leishmania species are morphologically indistinguishable. Species may be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies; however, these assays are not FDA cleared tests. In some of the studies (cutaneous or mucosal leishmaniasis), efforts were made to identify the Leishmania species by one of these tests. However, these efforts were for research purposes and details of the methods and performance characteristics of the assays in the laboratories where testing of clinical isolates was performed were not available for an independent review. Therefore, the clinical trial findings were based on cure rates in different geographic regions and not by Leishmania species. To support the treatment of mucosal leishmaniasis with miltefosine, one study was conducted in Bolivia known to be endemic for L. braziliensis. The efficacy and safety of miltefosine in other regions known to be endemic for L. guyanenesis or L. panamensis was not evaluated.

The following changes are recommended in the 'Indications and Usage' as well as 'Highlight' sections:

- *Visceral leishmaniasis in regions known to be endemic for Leishmania donovani.*
- *Cutaneous leishmaniasis in regions known to be endemic for Leishmania braziliensis, L. guyanenesis, L. panamensis.*
- *Mucosal leishmaniasis in regions known to be endemic for L. braziliensis.*

Otherwise, any reference to Leishmania species should be deleted.

- *In Section 14 'Clinical studies' the applicant has made statements such as* (b) (4) *Similar recommendations for not stating Leishmania species should be considered.*

6.3. FDA's version of the labeling

12.1 Mechanism of action
Miltefosine is an anti-leishmanial agent. *[see Clinical Pharmacology, Microbiology (12.4)]*

12.4 Microbiology
Mechanism of Action
The specific mode of action of miltefosine (b) (4) -against *Leishmania* species is unknown. The mechanism of action of miltefosine is likely to involve interaction with lipids (phospholipids and sterols), including membrane lipids, inhibition of cytochrome c oxidase (mitochondrial function), and apoptosis-like cell death.

Activity *In Vitro* and *In Vivo*
Miltefosine has (b) (4) -anti-leishmanial activity *in vitro* and in- (b) (4) -clinical infections [*see Clinical Studies (14)*]. Sensitivity of different *Leishmania* species as well as different strains of a *Leishmania* species to miltefosine may vary in different geographic regions.

Drug Resistance

(b) (4) –*In vitro* studies show a potential for development of resistance to miltefosine. Some strains of *L. braziliensis* with intrinsic resistance to miltefosine have been identified. However, t(b) (4)he clinical relevance of such an effect is not known.

Drug resistance could be due to a decrease in miltefosine accumulation within *Leishmania* parasite which is thought to be due to either an increase in drug efflux, mediated by the overexpression of the ABC transporter P-glycoprotein and/or a decrease in drug uptake by the inactivation of the miltefosine transport machinery that consists of the miltefosine transporter and its beta subunit. Mutation in the transporter gene was reported in a relapsed patient in one study.

[See appended electronic signature page]
Shukal Bala, Ph.D.
Microbiologist, DAIP

CONCURRENCE:
DAIP/Acting Microbiology Team Leader/ Kerry Snow MS, MT (ASCP)

CC:
NDA # 204684
DAIP/PM/Gregory DiBernardo

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/s/

SHUKAL BALA
09/26/2013

KERRY SNOW
09/26/2013

M E M O R A N D U M

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

DATE: 5 June 2013

TO: NDA 204684

FROM: Bryan S. Riley, Ph.D.
Acting Team Leader
OPS/New Drug Microbiology Staff

THROUGH: Stephen E. Langille, Ph.D.
Senior Review Microbiologist
OPS/New Drug Microbiology Staff

cc: Gregory DiBernardo
Regulatory Project Manager
OND/DAIP

SUBJECT: Product Quality Microbiology assessment of Microbial Limits for Impavido® Capsules [Re-Submission Date: 19 April 2013]

The Microbial Limits specification for Impavido® is acceptable from a Product Quality Microbiology perspective. Therefore, this submission is recommended for approval from the standpoint of product quality microbiology.

Impavido is a Capsule for oral administration.

The drug product is tested for Microbial Limits at release using a method consistent with USP Chapter <61> (Microbiological Examination of Non-sterile Products: Microbial Enumeration Tests) and <62> (Microbiological Examination of Non-sterile Products: Tests for Specified Microorganisms). The Microbial Limits acceptance criteria are consistent with USP Chapter <1111> (Microbiological Examination of Non-sterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use).

Table 1: Microbial Limits Specification

Test	Acceptance Criterion
Total Aerobic Count (USP <61>)	NMT (b) (4)
Total Yeast and Mold Count (USP <61>)	NMT (b) (4)

M E M O R A N D U M

The Microbial Limits test methods were verified to be appropriate for use with the drug product following procedures consistent with those in USP Chapter <61> and <62>.

The drug product will also be tested for Microbial Limits annually as part of the post-approval stability protocol.

ADEQUATE

Reviewer Comments – The microbiological quality of the drug product is controlled via a suitable testing protocol.

END

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/s/

BRYAN S RILEY
06/05/2013

STEPHEN E LANGILLE
06/05/2013

NDA Number: 204684 **Applicant: Paladin Therapeutics, Inc.** **Stamp Date:** 4/19/2013

Drug Name: Impavido (miltefosine) **NDA Type:** NME

On **initial** overview of the NDA application for filing:

	Content Parameter	Yes	No	Comments
1	Is the microbiology information (preclinical/nonclinical and clinical) described in different sections of the NDA organized in a manner to allow substantive review to begin?	X		
2	Is the microbiology information (preclinical/nonclinical and clinical) indexed, paginated and/or linked in a manner to allow substantive review to begin?	X		
3	Is the microbiology information (preclinical/nonclinical and clinical) legible so that substantive review can begin?	X		
4	On its face, has the applicant submitted *in vitro* data in necessary quantity, using necessary clinical and non-clinical strains/isolates, and using necessary numbers of approved current divisional standard of approvability of the submitted draft labeling?	X		
5	Has the applicant submitted any required animal model studies necessary for approvability of the product based on the submitted draft labeling?	X		
6	Has the applicant submitted all special/critical studies/data requested by the Division during pre-submission discussions?	X		
7	Has the applicant submitted the clinical microbiology datasets in a format which intents to correlate baseline pathogen with clinical and microbiologic outcome?			Some of the information requested was received by the Division on November 13, 2012.[1] However, the information was not provided for all the studies.
8	Has the applicant submitted draft/proposed interpretive criteria/breakpoint along with quality control (QC) parameters and interpretive criteria, if applicable, in a manner consistent with contemporary standards, which attempt to correlate criteria with clinical results of NDA/BLA studies, and in a manner to allow substantive review to begin?			N/A
9	Has the applicant submitted a clinical microbiology			N/A

[1] The applicant does not have details of the PCR method or the performance characteristics of the assay where testing was done for identifying *Leishmania* species. Use of PCR was an academic exercise.

	Content Parameter	Yes	No	Comments
	dataset in an appropriate/standardized format which intents to determine resistance development by correlating changes in the phenotype (such as *in vitro* susceptibility) and/or genotype (such as mutations) of the baseline pathogen with clinical and microbiologic outcome?			
10	Has the applicant used standardized or non-standardized methods for measuring microbiologic outcome? If non-standardized methods were used, has the applicant included complete details of the method, the name of the laboratory where actual testing was done and performance characteristics of the assay in the laboratory where the actual testing was done?			The applicant has used experimental tests (PCR, IFA, biochemical tests) for identifying *Leishmania* species. The applicant refers to some publications for details of the methods. However, the details of the methods for all the experimental tests and the performance characteristics of the assays in the laboratories where testing was performed were insufficient for review; such information was requested from the applicant. The applicant does not have details of the methods or the performance characteristics of the assays where testing was performed for identifying *Leishmania* species. Use of these experimental tests was an academic exercise (for details see meeting minutes dated 4/25/2013).
11	Has the applicant submitted draft labeling consistent with current regulation, divisional and Center policy, and the design of the development package?	X		
12	Has the applicant submitted annotated microbiology draft labeling consistent with current divisional policy, and the design of the development package?	X		
13	Have all the study reports, published articles, and other references been included and cross-referenced in the annotated draft labeling or summary section of the submission?	X		
14	Are any study reports or published articles in a foreign language? If yes, has the translated version been included in the submission for review?	X		

IS THE MICROBIOLOGY SECTION OF THE APPLICATION FILEABLE? Yes

If the NDA is not fileable from the microbiology perspective, state the reasons and provide comments to be sent to the Applicant.

N/A

Please identify and list any potential review issues to be forwarded to the Applicant for the 74-day letter.

- In response to Division request dated October 23, 2012, you provided us with the clinical microbiology information on November 12, 2012; this information included summary Tables for Studies 3154, 3168, Z020a, and Z020b as well as analysis data sets for Studies 3154 and 3168. However, the summary Tables for studies Z022 and Z025 and analysis data sets for studies Z020a, Z020b, Z022 and Z025 were not included. Please provide the following information for our review:
  - Please specify if there are any changes to the summary Tables and analysis datasets submitted in response to clinical microbiology requests on November 12, 2012.
  - Summary Tables for studies Z022 and Z025 (similar to those prepared for Studies Z022a and Z022b; see Table 1 for template).
  - Analysis datasets for studies Z020a, Z020b, Z022, and Z025 (see Table 2 for template) that will aid clinical microbiology review.

Shukal Bala	*06/04/2013*
Reviewing Microbiologist, DAIP	Date
Kerry Snow	*06/04/2013*
Acting Microbiology Team Leader, DAIP	Date

Table 1: Summary Table Template

Treatment Group/Species*	**End of Therapy n /N (%)**			**Follow-Up at 2 and 6 months n /N (%)**		
	Clinical Success	**Proven Parasitological Eradication!**	**Presumed Parasitologic Eradication**	**Clinical Success**	**Proven Parasitological Eradication!**	**Presumed Parasitologica Eradication**
ITT						
Miltefosine						
L. braziliensis						
Leishmania spp.						
Total						
Placebo or Comparator Drug**						
L. braziliensis						
Leishmania spp.						
Total						
PP						
Miltefosine						
L. braziliensis						
Leishmania spp.						
Total						
Placebo or Comparator Drug**						
L. braziliensis						
Leishmania spp.						
Total						

* Specify the *Leishmania* species that would be applicable for the study

** Please add the control arm data (placebo, active control) as appropriate for the trial

! If parasitological testing was not performed, please state not done (ND)

Table 2: Clinical Microbiology Dataset Template

PtID	Center	Trt Group	Duration of Trt	ITT/PP Flag	Phase	Specimen Source/ Lab	Test used for species identification results by lab				Clinical Response	Parasitological Response	Status of *Leishmania* infection (Relapse/New/ Unresponsive)*
							Stain*	Culture*	PCR*	IFA*			
101		M			Baseline	Aspirate							
101		M			2 weeks after the last dose	Aspirate							
101		M			2 months after the last dose	Smear							
101		M			6 months after the last dose	Aspirate							
102		M			Baseline	Smear							
102		M			2 weeks after the last dose	Smear							
102		M			6 months after the last dose	Smear							
103		P			Baseline	Biopsy							
103		P			2 weeks after the last dose	Biopsy							
103		P			6 months after the last dose	Biopsy							
103		P			Baseline	Aspirate							
103		P			2 weeks after the last dose	Aspirate							
103		P			6 months after the last dose	Aspirate							

* If parasitological testing by staining, culture, PCR, or IFA was not collected then these columns can be deleted
M= miltefosine; P = placebo

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/s/

SHUKAL BALA
06/04/2013

KERRY SNOW
06/04/2013

MICROBIOLOGY FILING CHECKLIST FOR NDA 204684

NDA Number: 204684 **Applicant: Paladin Therapeutics, Inc.** **Stamp Date:** 9/27/ 2012

Drug Name: Impavido (miltefosine) **NDA Type:** NME (1)

On **initial** overview of the NDA application for filing:

	Content Parameter	Yes	No	Comments
1	Is the microbiology information (preclinical/nonclinical and clinical) described in different sections of the NDA organized in a manner to allow substantive review to begin?	X		
2	Is the microbiology information (preclinical/nonclinical and clinical) indexed, paginated and/or linked in a manner to allow substantive review to begin?	X		
3	Is the microbiology information (preclinical/nonclinical and clinical) legible so that substantive review can begin?	X		
4	On its face, has the applicant submitted *in vitro* data in necessary quantity, using necessary clinical and non-clinical strains/isolates, and using necessary numbers of approved current divisional standard of approvability of the submitted draft labeling?	X		
5	Has the applicant submitted any required animal model studies necessary for approvability of the product based on the submitted draft labeling?	X		
6	Has the applicant submitted all special/critical studies/data requested by the Division during pre-submission discussions?	X		
7	Has the applicant submitted the clinical microbiology datasets in a format which intents to correlate baseline pathogen with clinical and microbiologic outcome?		X	• Parasitological results (*Leishmania* species) identified not included in the datasets[1] • Analysis datasets not included[1]
8	Has the applicant submitted draft/proposed interpretive criteria/breakpoint along with quality control (QC) parameters and interpretive criteria, if applicable, in a manner consistent with contemporary standards, which attempt to correlate criteria with clinical results of NDA/BLA studies, and in a manner to allow			N/A

[1] Information request was communicated to the applicant on October 23, 2012. For details see Appendix-1. The applicant responded in letter dated November 12, 2012 (SDN-004) to the comments and provided some of the information requested that includes *Leishmania* species and analysis data sets for some of the studies. The applicant states that details of the parasitological methods will be provided but does not specify the time frame by which response should be expected.

MICROBIOLOGY FILING CHECKLIST FOR NDA 204684

	Content Parameter	Yes	No	Comments
	substantive review to begin?			
9	Has the applicant submitted a clinical microbiology dataset in an appropriate/standardized format which intents to determine resistance development by correlating changes in the phenotype (such as *in vitro* susceptibility) and/or genotype (such as mutations) of the baseline pathogen with clinical and microbiologic outcome?			N/A
10	Has the applicant used standardized or nonstandardized methods for measuring microbiologic outcome? If nonstandardized methods were used, has the applicant included complete details of the method, the name of the laboratory where actual testing was done and performance characteristics of the assay in the laboratory where the actual testing was done?			The sponsor has used PCR, IFA, biochemical tests for identifying *Leishmania* species. However, the details of the methods for experimental tests and the performance characteristics of the assay in the laboratories where testing was performed could not be found in the NDA submission[1]
11	Has the applicant submitted draft labeling consistent with current regulation, divisional and Center policy, and the design of the development package?	X		
12	Has the applicant submitted annotated microbiology draft labeling consistent with current divisional policy, and the design of the development package?	X		
13	Have all the study reports, published articles, and other references been included and cross-referenced in the annotated draft labeling or summary section of the submission?	X		
14	Are any study reports or published articles in a foreign language? If yes, has the translated version been included in the submission for review?	X		

IS THE MICROBIOLOGY SECTION OF THE APPLICATION FILEABLE? Yes

If the NDA is not fileable from the microbiology perspective, state the reasons and provide comments to be sent to the Applicant.

N/A

MICROBIOLOGY FILING CHECKLIST FOR NDA 204684

Please identify and list any potential review issues to be forwarded to the Applicant for the 74-day letter.

1. You state in your letter dated November 12, 2012, that a summary of the parasitological methods used in different laboratories will be provided. In addition to the details of the methods, please include the performance characteristics of the assays in the laboratory where testing was performed. The name and address of the laboratories should be specified.

Shukal Bala	*11/21/12*
Reviewing Microbiologist	Date
Lynette Berkeley	*11/21/12*
Microbiology Team Leader	Date

MICROBIOLOGY FILING CHECKLIST FOR NDA 204684

Appendix -1

The following comments were communicated to the applicant on October 23, 2012:

- Parasitology data were included for only two clinical studies (Z020b and Z022) and the results were presented as positive by either microscopy or PCR. For Study Z020b, *Leishmania* species identified was only specified for some of the patients. Please include parasitology data including *Leishmania* species, by test, identified for all patients in all the datasets.

- Please format the clinical microbiology datasets including all of the above in one SAS Transport file as shown in the attached Table 1 for each of the studies (3154, Z025, 3168, Soto, Z020b, and Z022). Please note that Table 1 is based on study report 3168. The *Leishmania* species will vary based on the country where the trial was performed. Additionally, the tests used for parasitological response may vary in different laboratories. Therefore, changes to the Table should be made as appropriate.

- Submit summary Tables (as shown in Table 2) of the results by baseline *Leishmania* species in the treatment arms. Patients with a single baseline pathogen and those patients with mixed infection should be shown separately. A separate summary Table should be included for each study.

- Submit details of all the parasitological methods used for identification of *Leishmania* species as well as for measuring parasitologic response at follow up visits (e.g., 2 weeks, 2 months and 6 months after end of treatment for Study 3168). Other than microscopy and culture, it appears that all the other tests used such as PCR and immunofluorescent testing are not FDA cleared and considered experimental assays. Please clarify. As discussed in the meeting held on January 13, 2012 (for details see meeting minutes dated February 9, 2012) if the tests are FDA cleared, then the test brochure should be provided for our review. If these are experimental assays, then details of the methods used in the laboratory where testing was performed and performance characteristics of the assays including appropriate quality control measures in that laboratory should be provided for an independent review.

- It appears that photographs were taken of the parasitological findings. It will be helpful if some of these are included in the submission.

- It also appears that cytokines were measured in some of the studies such as Z027. Please provide details of the methods used and the results for our review.

Table 1: CL Clinical microbiology dataset sample template

PtID	Center (Colom /Gaut)	Trt Grp	Durat of trt	ITT/PP F	Phase	Specimen Source/Lab	Test used for species identification ar results by lab				Clinical Response	Lesion Size	Parasitolo Response	Status of Leishmania (Relap/New/ onsive)
							Stain*	Culture	PCR	IFA				
101		M			Baseline	Aspirate								
101		M			2 weeks after dose	Aspirate								
101		M			2 mths after t dose	Smear								
101		M			6 mths after t dose	Aspirate								
102		M			Baseline	Smear								
102		M			2 weeks after dose	Smear								
102		M			6 mths after t dose	Smear								
103		P			Baseline	Biopsy								
103		P			2 weeks after dose	Biopsy								
103		P			6 mths after t dose	Biopsy								
103		P			Baseline	Aspirate								
103		P			2 weeks after dose	Aspirate								
103		P			6 mths after t dose	Aspirate								

*If different stains were used then those should be specified; If more than one species was identified in a specimen from a patient then that should be identified
M= miltefosine; P = placebo

MICROBIOLOGY FILING CHECKLIST FOR NDA 204684

Table 2: Summary Tables by the method used (stain, PCR, culture, etc):

Treatment Group/Species	**End of Therapy n /N (%)**			**Follow-Up at 2 and 6 months n /N (%)**		
	Clinical Success	**Proven Parasitologic Eradication**	**Presumed Parasitologic Eradication**	**Clinical Success**	**Proven Parasitologic Eradication**	**Presumed Parasitologic Eradication**
ITT						
Miltefosine						
Colombia						
L. vianna panamensis						
Guatemala						
L. v. braziliensis						
L. mexicana mexicana						
Total						
Placebo						
Colombia						
L. vianna panamensis						
Guatemala						
L. v. braziliensis						
L. mexicana mexicana						
Total						
PP						
Miltefosine						
Colombia						
L. vianna panamensis						
Guatemala						
L. v. braziliensis						
L. mexicana mexicana						
Total						
Placebo						
Colombia						
L. vianna panamensis						
Guatemala						
L. v. braziliensis						
L. mexicana mexicana						
Total						

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/s/

SHUKAL BALA
11/21/2012

LYNETTE Y BERKELEY
11/24/2012