Van Bocxlaer, K; Yardley, V; Murdan, S; Croft, SL; (2016) Topical formulations of miltefosine for cutaneous leishmaniasis in a BALB/c mouse model. The Journal of pharmacy and pharmacology, 68 (7). pp. 862-72. ISSN 0022-3573 DOI: https://doi.org/10.1111/jphp.12548

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Informative title: Topical formulations of miltefosine for cutaneous leishmaniasis in a BALB/c mouse model

Authors: Katrien Van Bocxlaer1,2, Vanessa Yardley2, Sudaxshina Murdan1, Simon L. Croft2

1 UCL School of Pharmacy, Department of Pharmaceutics, 29-39 Brunswick Square, London, WC1N 1AX, UK
2 London School of Hygiene & Tropical Medicine, Department of Infection and Immunology, Keppel Street, London, WC1E 7HT, UK

Corresponding author: Simon L. Croft, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom; simon.croft@lshtm.ac.uk, phone: +44 (0)20 7927 2601, fax: +44 (0)20 7927 2739

Abstract

Cutaneous leishmaniasis (CL) is caused by several species of the protozoan parasite *Leishmania* and affects approximately 10 million people worldwide. Currently available drugs are not ideal due to high cost, toxicity, parenteral administration and suboptimal efficacy. Miltefosine is the only oral treatment (Impavido®) available to treat CL, given over a period of 28 days with common side effects such as vomiting and diarrhoea.

Objective. To explore the local application of miltefosine as a topical formulation to enhance activity and reduce the drug’s adverse effects.

Methods. The anti-leishmanial activity of miltefosine was confirmed in vitro against several *Leishmania* species. The permeation of miltefosine, in different solvents and solvent combinations, through BALB/c mouse skin was evaluated in vitro using Franz diffusion cells. The topical formulations which enabled the highest drug permeation or skin disposition were tested in vivo in BALB/c mice infected with *L. major*.

Results. The overall permeation of miltefosine through skin was low regardless of the solvents used. This was reflected in limited anti-leishmanial activity of the drug formulations when applied topically in vivo. All topical formulations caused skin irritation.

Conclusions. We conclude that miltefosine is not an appropriate candidate for the topical treatment of CL.

Keywords: miltefosine, cutaneous leishmaniasis, topical formulation, drug delivery, skin.
Introduction

The leishmaniases are poverty-related diseases caused by up to 20 Leishmania species [1] that are transmitted by sandflies. With 220 000 new cases a year, cutaneous leishmaniasis (CL) is the most common form of leishmaniasis [2]. The presence of the Leishmania parasites in macrophages in the skin dermis causes a range of clinical symptoms, from small nodules to large plaques and disfiguring ulcers. Drugs currently administered to treat CL are mainly repurposed with sub-optimal efficacy attributable to (i) differences in drug susceptibility between the different Leishmania species which can lead to sub-therapeutic drug concentrations [3, 4], (ii) the failure of the drug to reach the target tissue, (iii) adverse effects [5] and being not patient-friendly [6]. Currently there is no safe treatment that is guaranteed to cure CL.

Miltefosine (Figure 1) is an anti-leishmanial drug that is used as an oral formulation for the treatment of visceral leishmaniasis (VL) [7, 8] and in several countries to treat CL [9]. The most frequently reported side effects are (i) gastro-intestinal discomfort that is often the cause of poor compliance to therapy [10], (ii) teratogenicity that calls for adequate contraception throughout the treatment of young females, and (iii) hepat- and nephrotoxicity requiring patient monitoring [11]. A topical miltefosine treatment would offer certain advantages over systemic treatment. The formulation, directly applied to the target site, would avoid or at least reduce potential side effects of systemic exposure, require less intensive patient follow up and would also improve patient compliance. As miltefosine is already approved for clinical use, reformulating miltefosine into a topical treatment could provide a more cost effective route for treatment development compared to a 'de novo' drug discovery process especially in an area with minimal financial incentive.

To permeate the stratum corneum, the main barrier to permeation for most topically applied drugs, an active compound should ideally have a low molecular weight (< 500 g/mol) [12], less than three H-bond donors [13], good solubility in the formulation vehicle and a partition coefficient between one and three [14, 15]. The physico-chemical properties of miltefosine (Figure 1), are indicative of potential skin permeation. Moreover, recent studies have shown enhanced permeation of drugs, particularly drugs with high water solubility, through Leishmania-infected mouse skin [16] and a topical miltefosine solution (Miltex®) has been used to treat superficial metastases of skin cancer, indicating some drug permeation. However, miltefosine is an amphiphilic and zwitterionic molecule at skin pH (pH 5.5) containing both a positive and a negative charge (pKa ≈ 2) [17] and behaves as a surfactant. The inherent aqueous solubility of such compounds challenges permeation through lipophilic membranes such as stratum corneum.
Our aim therefore was to systematically investigate \textit{for the first time} the potential of miltefosine as a topical anti-leishmanial drug. We evaluated (i) the activity of miltefosine against a range of intracellular \textit{Leishmania} amastigotes \textit{in vitro}, (ii) the permeation of miltefosine when applied to BALB/c mouse skin in a range of solvents (propylene glycol (PG), water, dimethyl isosorbide (DMI) and octyl salicylate (OSAL)) using Franz diffusion cells, (iii) the potential enhancement of \textit{in vitro} permeation using a combination of these solvents and (iv) the \textit{in vivo} anti-leishmanial activity of the optimal solvent systems upon topical administration in BALB/c mice. \textit{The solvents were chosen because (i) they had a broad range of physicochemical properties, (ii) they have been reported to enhance percutaneous drug delivery (Table 1), and (iii) they have been approved by the FDA as inactive ingredient for drug products [18].}

\textbf{Materials and Methods}

\textbf{Materials}

Miltefosine was donated by Paladin Labs Inc (Montréal, Canada). The [\textsuperscript{14}C]-miltefosine (specific activity: \textsuperscript{36}mCi/mmol, concentration: \textsuperscript{900}uCi/ml, radiochemical purity: 98.3\%) was from Nycomed Amersham Pharmacia (Buckinghamshire, UK). Schneider’s insect medium, RPMI-1640 and M199 medium were obtained from Sigma Aldrich (Dorset, UK). Foetal calf serum was from Harlan Laboratories (Derbyshire, UK) and heat-inactivated by exposure to 56°C for 30 minutes. Propylene glycol (PG), dimethyl isosorbide ether (DMI), octyl salicylate (OSAL) and phosphate buffered saline (PBS) tablets were from Sigma Aldrich (UK). The Franz diffusion cells, diameter of 0.90±0.03 cm and mean receptor volume of 2.65±0.06 ml, were obtained from Soham Scientific (Fordham, UK). The Optiphase™ supermix and Hionic Fluor™ liquid scintillation cocktails and Solvable™ were purchased from Perkin Elmer (Coventry, UK). Liposomal amphotericin B (AmBisome®, Gilead Sciences, USA) and amphotericin B deoxycholate (Fungizone®, ER Squibb, UK) were reconstituted according to manufacturer’s instructions.

\textbf{Leishmania} parasites, cell culture and animals

Promastigotes of \textit{L. major} (MHOM/SA/85/JISH118); \textit{L. panamensis} (MHOM/PA/67/BOYNTON); \textit{L. aethiopica} (MHOM/ET/84/KH); \textit{L. mexicana} (MNYC/ BZ/62/M379) and \textit{L. tropica} (MHOM/IR/2013/HTD4) were taken from liquid nitrogen stocks. \textit{L. panamensis} was cultured in M199 medium plus 10\% heat-inactivated fetal calf serum (HiFCS) at 26°C while the other four species were cultured in Schneider’s insect medium supplemented with 10\% HiFCS.

Female BALB/c mice (6-8 weeks old) were purchased from Charles River (Margate, UK) and housed in a controlled environment of 55\% relative humidity and 26°C. They were provided with tap water and
a standard laboratory diet. All animal experiments were approved by the Animal Welfare and Ethical Review Board of the London School of Hygiene & Tropical Medicine and carried out under UK home office licence (PPL 70/8207) according to the Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU.

Evaluation of the anti-leishmanial activity of miltefosine against intracellular amastigotes

Peritoneal exudate macrophages (PEMs) were harvested by lavage 24 hours after starch induction (2% aq) from female CD-1 mice (LSHTM in house colony). The cells were seeded in 16-well Lab-Tek™ slides in RPMI-1640 supplemented with 10% HiFCS at a density of 4x10⁴ per well (100 μl). After 24 hours incubation at 37°C and 5% CO₂/95% air mixture, the adhered PEMs were infected with stationary phase promastigotes at a ratio of 3:1 (for L. tropica and L. major) or 5:1 (for L. mexicana, L. aethiopica and L. panamensis) promastigotes and maintained at 34°C in a 5% CO₂/95% air mixture. 24 Hours later, the infection was checked and the overlay replaced with medium-containing drug, in quadruplicate.

Final miltefosine concentrations were 30, 10, 3 & 1 μM. Amphotericin B (Fungizone®) was included as control drug. After 72 hours incubation, all the slides were methanol-fixed and Giemsa-stained. Drug activity was determined microscopically by counting the number of infected macrophages in drug-treated cultures compared to untreated cultures. The EC50 and EC90 values were calculated by non-linear sigmoidal curve fitting (variable slope) using Prism Software (GraphPad, UK).

Exploration of topical drug delivery systems for miltefosine

Drug solubility. The saturated solubility of miltefosine in PG, DMI, OSAL and water was determined as described by [19]. Drug concentrations were determined by LC-MS (Finnigan LCQ, analytical laboratory, LSHTM).

Solvent miscibility. In order to deliver the drug from a stable solvent system, the compatibility of solvents was explored in binary and ternary phase miscibility studies. The appropriate volume and ratio of solvents (v/v) were added into vials that were vortexed for two minutes and left to stand at room temperature. After one hour, the samples were marked as immiscible (when phase separation was visible) or miscible (when a homogeneous phase was seen). Ternary phase diagrams were drawn using the OriginPro software (Northampton, UK).

Permeation and disposition of miltefosine in BALB/c mouse skin. The test formulations contained 6% (w/v) miltefosine (same as Miltex®) in the appropriate vehicle consisting of a single, binary or ternary solvent mixture. After addition of miltefosine, the mixture was stirred overnight at 32°C. Before application to the skin, the drug formulations were spiked with [14C]-miltefosine to a final concentration of 4 μCi/ml and vortexed for two minutes.
On the day of the permeation experiment, the mice were sacrificed and full-thickness dorsal skin was removed, and cut to circular discs that were mounted between the donor and receptor compartments of the Franz diffusion cells. The receptor fluid, PBS (pH 7.4), was sonicated and placed into the receptor compartment together with a magnetic stirrer and the cells were incubated in a warm water bath (32°C). After one hour, 500 µl of the test solution was applied to each donor compartment after which 200 µl of receptor phase was removed and replaced with fresh PBS at regular time intervals over a duration of 48 hours.

For the quantification of miltefosine, 100 µl of the receptor sample was transferred to a 96-well flexible MicroBeta plate (Perkin Elmer, UK) and 100 µl of Optiphase™ supermix was added. A standard curve was prepared by double-diluting the donor solution in PBS for PG and water and in methanol for DMI and OSAL, as the latter two solvents are not miscible with water. Blanks consisted of 100 µl PBS or methanol and 100 µl of Optiphase™ supermix. Scintillation counting was conducted using a Microbeta2 plate reader equipped with 2 detectors (Perkin Elmer, UK). The cumulative amount of drug permeated per surface area of skin was plotted as a function of time. The slope and thus flux was calculated by linear regression of the data points obtained between 20 and 36 hours following topical drug application.

After 48 hours, the permeation experiment was terminated and mass balance studies were conducted as follows. The donor solution was transferred into a clean vial and any left overs were removed from the skin by carefully wiping the surface with a cotton swab, followed by repeatedly pipetting with one ml of a methanol:water (3:7 (v/v)) solution. This was repeated three times. To extract the miltefosine absorbed in the cotton, one ml of a methanol:water (3:7 (v/v)) solution was added to the vial containing the swab, and the mixture was left on a shaking plate for five hours, after which 100 µl of the liquid was transferred to a flexible Microbeta 24-well plate. To quantify the amount of miltefosine, 400 µl of Hionic-fluor™ scintillation fluid was added and left to acclimatize before reading with a Microbeta2 plate reader. For each formulation tested, a standard curve of 12 serial double dilutions was included and each plate also contained three methanol:water blanks. The amount of miltefosine in each sample was calculated from the miltefosine standard curve.

To determine the amount of miltefosine in the skin, the skin and one ml of Solvable™ were transferred to a vial, incubated at 50°C and vortexed regularly until a homogenous mixture was obtained. A sample of this homogenate (100 µl) was mixed with Hionic-fluor™ (300 µl) in a microbeta plate and analysed using the Microbeta2 plate reader. Controls (in triplicate) included untreated skin spiked with a known amount of radiolabelled miltefosine to confirm no drug breakdown occurred during the extraction.
procedure, and skin unexposed to any drug to correct for effects due to skin components. The amount of miltefosine in each sample was calculated using the miltefosine standard curve.

**In vivo evaluation of the anti-leishmanial activity of topical formulations**

60 Female BALB/c mice were subcutaneously injected with 2x10⁷ stationary phase *L. major* JISH118 promastigotes (200 µl) on the rump above the tail. Approximately seven days post infection, small nodules were visible. When the nodule reached a diameter of 3-4 mm, the mice were randomly allocated to nine groups of five mice to test the drug formulations, and five groups of three mice to test the effect of the solvent (without miltefosine).

Five different formulations were chosen based on the outcome of the permeation and mass balance study. They were 6% (w/v) miltefosine in water, in PG, in OSAL, in DMI and in OSAL-DMI (1:1). For the groups receiving a topical formulation (or controls) 50 µl was applied to the lesion twice daily. One group received miltefosine (in water) orally at a dose equivalent to 20mg/kg once a day. AmBisome® (25 mg/kg) was administered intravenously every other day. Formulations were administered over a period of 5 days except for the groups receiving AmBisome® or miltefosine orally; these received treatment over a duration of 10 days.

The efficacy of the formulation was evaluated by assessing (i) rate of change of lesion size and (ii) parasite load. The lesion diameter was measured daily in 2 perpendicular directions using digital callipers and the average diameter was plotted as a function of time. Three days after the end of drug administration, the parasite load was determined by counting the amastigotes microscopically after homogenisation of the whole lesion in 1ml of sterile PBS.

**Statistical analyses.** The EC50 and EC90 values were calculated by non-linear sigmoidal curve fitting (variable slope) using Prism Software (GraphPad, UK). The results of the *in vitro* permeation and the *in vivo* parasite load post-treatment were evaluated for statistical differences using a one-way ANOVA post hoc Tukey test (SPSS software, version 19.0), while the lesion size progression among the groups was evaluated using repeated measures ANOVA. SPSS software, version 19.0 was used for all analyses and differences were considered statistically significant at p<0.05.

**Results**

**In vitro anti-leishmanial activity of miltefosine**

Miltefosine exhibited variable activity against a panel of *Leishmania* parasites as shown in Table 2. EC50 and EC90 values ranged from 7.8 µM to 45.9 µM and 19.5 µM to 166.3 µM respectively. When ranking the activity, miltefosine was most active against *L. aethiopica > L. tropica > L. panamensis > L.*
major > L. mexicana. Amphotericin B, included as control drug, was highly active (in the nanomolar range) against all species.

**Saturated solubility of miltefosine in the solvents and solvent miscibility**

Miltefosine was highly soluble in water and PG (at 440 and 738 mg/ml respectively), while its solubility in OSAL and DMI (at 36 and 5 µg/ml respectively) was approximately four to five orders of magnitude lower.

Miscibility of binary and ternary solvent mixtures are shown in Table 3 and Figure 2 respectively. PG, water and DMI are miscible when combined in binary systems and hence the influence of combinations of these solvents on the permeation of miltefosine were tested in permeation studies. OSAL had limited miscibility with other solvents, being miscible with only DMI at a ratio of 1:1; this binary mixture was also evaluated in permeation studies. To ensure miscibility throughout the experiment, two ternary phase systems away from the miscible/immiscible boundary were selected. These included OSAL-DMI-PG (2:5:3) and H₂O-DMI-PG (3:4:3). An OSAL-DMI-H₂O mixture was not included due to limited miscibility.

**Influence of solvents on the In vitro permeation of miltefosine**

The permeation profiles of the formulations tested (Table 4) are shown in Figure 3. When the influence of single solvents on miltefosine permeation was analysed, it was seen that miltefosine did not permeate the skin when dissolved in PG, in contrast to permeation from water, DMI or OSAL. There was no statistically significant difference in flux when the drug was applied in water, DMI and OSAL (Table 5, p>0.05).

Based on these initial findings, the influence of four miscible binary and two ternary solvent mixtures on miltefosine permeation through skin was tested. During the first 36 hours of permeation, miltefosine only permeated BALB/c mouse skin when applied in DMI-OSAL (1:1). There was no significant difference in flux when compared to the single solvent mixtures water, DMI and OSAL (Table 5, p>0.05). The lag time and permeability coefficient for this binary solvent formulation were, however, significantly lower compared to those of the single solvent formulations (p<0.05).

The mass balance studies showed that only a very small fraction of the applied drug (< 4%) had permeated through the skin from all formulations over 48 hours. Beyond 36 hours, an increase in permeation was seen for most formulations in particular for the miltefosine in water formulation. This was not unexpected as surfactants and particularly ionic surfactants such as miltefosine are known skin irritants and have been reported to damage the skin [20-23], which would result in enhanced
permeation. Total drug recovery ranged from 76%-102% (Figure 4) with most of the applied drug remaining on the skin surface. Miltefosine could not be detected in the skin when applied in PG, H2O-DMI, or the two ternary phase solvent systems. For the other six formulations, only small fractions of the applied miltefosine ranging from 0.5-1.4% were found in the skin, with no significant difference among the formulations tested (p<0.05).

In vivo anti-leishmanial activity

Four formulations that demonstrated skin permeation in vitro i.e. 6% miltefosine (w/v) in water, DMI, OSAL and DMI-OSAL (1:1), were tested in vivo against experimental CL. The lesion size in the experimental groups progressed at the same rate as the untreated control (Figure 5, p>0.05) indicating no in vivo efficacy of topical miltefosine. However, the topical application of both control and drug formulations was halted after five days due to skin irritation. In contrast, the positive control, intravenous AmBisome® reduced the size of the lesion significantly (p<0.05).

When the parasite load in the lesions was compared (Figure 6), no clear trend emerged. Of the treated groups, mice receiving AmBisome® showed a statistically significantly lower parasite burden compared to the groups receiving oral miltefosine, topical miltefosine in OSAL, topical DMI only and OSAL only (p<0.05). No single group had a significantly lower parasite load than the untreated control (p>0.05).

Discussion

Miltefosine is a recommended oral treatment for both cutaneous and visceral leishmaniasis. We confirmed the in vitro activity of miltefosine against a panel of species that cause CL with similar activities to previously reported [24, 25]. Only the EC50 values for L. mexicana were higher, probably due to the different strain used. Overall miltefosine was slightly less active against L. mexicana and L. major, which was not unexpected as difference in intrinsic sensitivity to miltefosine across Leishmania species is known [3, 24].

In vitro permeation assays showed limited permeation of miltefosine across full-thickness mouse skin even when applied in different solvents or solvent mixtures. This was not unexpected as the stratum corneum consists of dead cells surrounded by lipids, expecting to hinder the diffusion of hydrophilic molecules such as miltefosine. Moreover, Miltex®, a topical solution of 6% miltefosine (w/v) in propylene glycol ethers was indicated for cutaneous metastases of breast cancer with limited depth [26] possibly due to the ability of miltefosine to permeate into the superficial layers of the skin where
the cancer cells are situated, while it is unable to reach the dermis where *Leishmania* amastigotes reside inside macrophages.

Different solvents were used to enhance the permeation of miltefosine into the skin. Miltefosine demonstrated a higher saturated solubility in water and PG compared to DMI and OSAL. This is important as the saturation level of a drug in its formulation is positively related to the thermodynamic activity, the driving force for permeation [27, 28]. In fact, the permeation of miltefosine was slightly higher when applied in DMI or OSAL because these formulations were suspensions and consequently, the thermodynamic activity of the active compound in its vehicle, was maximal and equal to one. In contrast, no permeation was observed when miltefosine was applied in PG, the solvent in which miltefosine demonstrated the highest solubility. There was some permeation when miltefosine was applied in water, however, the high solubility of miltefosine in both solvents resulted in a lower thermodynamic activity and thus a lower permeation. Furthermore, the permeability coefficient ($K_p$) for the drug formulation in water was statistically significantly lower compared to $K_p$ for the DMI and OSAL formulation. This indicated that miltefosine when formulated in water had a low affinity for the skin but a high affinity for the vehicle which was reflected by the high solubility of miltefosine in water. Moreover the high permeability coefficients for miltefosine in DMI and OSAL indicated a high affinity for the skin and favoured partitioning into the skin. Previous studies reported an enhanced percutaneous drug permeation when binary and even ternary miscible solvent systems were used compared to single solvents [29-34]. A combination of solvents, however, does not always result in an additional increase of drug permeation [35, 36]. Our results show no enhanced permeation of miltefosine when solvent mixtures were used. In fact, the flux obtained using the binary OSAL-DMI combination was 40 times lower than the flux obtained when using OSAL and DMI alone.

The overall low permeation of miltefosine upon topical application is probably why the formulations were unable to cure CL in BALB/c mice as indicated by an increasing lesion size and a high parasite load. In contrast to our results, Schmidt-Ott et al reported that Miltex®, which also contained 6% miltefosine, cured CL lesions due to *L. major* and *L. mexicana* upon topical application [37]. However, these results were not reproducible when conducted in our lab (Yardley and Croft, unpublished data), even though the activity of miltefosine against a range of *Leishmania* species was confirmed [24].

Two clinical trials were conducted to evaluate the efficacy of topical Miltex® against CL (Bachmann P., unpublished data). One trial was conducted in Syria and included 16 patients with nodular CL who applied the formulation twice daily. The other trial was conducted in Colombia where the 19 involved patients received treatment once a day for 4 weeks [38]. Both trials of topically applied Miltex® were
unable to show efficacy against CL, even though oral administration has been shown to cure CL in patients [4, 39].

In this study we observed that oral miltefosine (20 mg/kg/day) was also unable to reduce the lesion size or the parasite burden in the skin of mice, confirming a previous study that showed no significant lesion size reduction with a similar dose of miltefosine although higher doses, with toxic side effects, did reduce lesion size and parasite burden [40]. In contrast, in a clinical trial in Iran, oral miltefosine was shown to be effective against CL caused by *L. major* with cure rate of about 81% [41].

Additionally, we observed that the lesion size results did not correlate with the parasite load per lesion. A possible explanation could be (i) the skin irritation that exaggerated the lesion size readings by enhancing the lesion size and (ii) the large variation in parasite burdens observed per group. Moreover, severe skin irritation observed in the groups receiving miltefosine topically, required an early halt to dosing. This was not surprising as surfactants such as miltefosine have been reported to damage skin. Research has shown protein denaturation [20], swelling of the stratum corneum [21] and lipid depletion and solubilisation in the stratum corneum [22]. Additionally, OSAL was reported to be mildly irritating when applied to rabbit skin which could have caused further damage to the skin [42].

*It is unclear how our results would translate to disease in humans. First, there are physical differences between human and mouse skin for example full-thickness mouse [43]. Furthermore the number of hair follicles and differences in the composition of intercellular SC lipids [44] also contribute to the overall higher permeability of mouse skin compared to human skin [45-47].*

**Conclusion**

Although miltefosine showed activity against a panel of *Leishmania* parasites in vitro, this did not translate into *in vivo* activity when tested in topical formulations against experimental CL in mice. *In vitro* Franz diffusion cell studies showed poor drug permeation into and through the skin, suggesting that miltefosine probably did not reach the parasites that reside in the dermal layer of the skin. Moreover after 5 days of in vivo application, all treatments including the formulation that contained water as vehicle, had caused significant irritation and drug application had to be stopped. We conclude that miltefosine is not an appropriate candidate for topical treatment for CL.

**Conflict of Interest**

The authors state no conflict of interest.

**Acknowledgements**

Katrien Van Bocxlaer was supported by a Bloomsbury Colleges PhD studentship.
References


37. Schmidt-Ott, R., et al., *Topical treatment with hexadecylphosphocholine (Miltefl) efficiently reduces parasite burden in experimental cutaneous leishmaniasis. 1999(0035-9203 (Print)).


Table 1. The four selected solvents, dimethyl isosorbide, octyl salicylate, propylene glycol and water and their physicochemical properties.

<table>
<thead>
<tr>
<th></th>
<th>Dimethyl isosorbide</th>
<th>Octyl salicylate</th>
<th>Propylene glycol</th>
<th>Water</th>
</tr>
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<tbody>
<tr>
<td>Mol. Wt. (g/mol)</td>
<td>174</td>
<td>250</td>
<td>76</td>
<td>18</td>
</tr>
<tr>
<td>Log K&lt;sub&gt;O/W&lt;/sub&gt;</td>
<td>0.07</td>
<td>5.97</td>
<td>-1.06</td>
<td>-1.38</td>
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<tr>
<td>Solubility parameter (cal/cm&lt;sup&gt;3&lt;/sup&gt;)&lt;sup&gt;1/2&lt;/sup&gt;</td>
<td>9.97</td>
<td>10.87</td>
<td>14.07</td>
<td>22.97</td>
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<tr>
<td>Density at 25°C (g/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.16</td>
<td>1.1</td>
<td>1.04</td>
<td>1.0</td>
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<tr>
<td>Penetration enhancement</td>
<td>[48]</td>
<td>[49-51]</td>
<td>[52, 53]</td>
<td>[54, 55]</td>
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</table>
Table 2. *In vitro* anti-leishmanial activity as determined by microscopic counting of *Leishmania* infected macrophages treated with miltefosine (30, 10, 3.3 and 1.1 uM; n= number of experiments).

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Amphotericin B</th>
<th>Miltefosine</th>
</tr>
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<tr>
<td></td>
<td>EC$_{50}$ (µM) (95% CI)</td>
<td>EC$_{50}$ (µM)</td>
<td>EC$_{50}$ (µM) (95% CI)</td>
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<tr>
<td><em>L. tropica</em></td>
<td>1</td>
<td>0.07 (0.06-0.07)</td>
<td>0.29</td>
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<tr>
<td></td>
<td>2</td>
<td>0.08 (0.08-0.09)</td>
<td>0.30</td>
</tr>
<tr>
<td><em>L. major</em></td>
<td>1</td>
<td>0.12 (0.11-0.14)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.05 (0.04-0.06)</td>
<td>-</td>
</tr>
<tr>
<td><em>L. aethiopica</em></td>
<td>1</td>
<td>0.12 (0.11-0.12)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.11 (0.10-0.12)</td>
<td>0.24</td>
</tr>
<tr>
<td><em>L. mexicana</em></td>
<td>1</td>
<td>0.43 (0.39-0.46)</td>
<td>1.10</td>
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<tr>
<td></td>
<td>2</td>
<td>0.69 (0.55-0.69)</td>
<td>1.21</td>
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<tr>
<td><em>L. panamensis</em></td>
<td>1</td>
<td>0.14 (0.13-0.16)</td>
<td>0.29</td>
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<tr>
<td></td>
<td>2</td>
<td>0.12 (0.09-0.14)</td>
<td>0.15</td>
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Table 3. Miscibility of binary solvent mixtures (1:1 ratio, ✓ miscible; × immiscible).

<table>
<thead>
<tr>
<th></th>
<th>OSAL</th>
<th>DMI</th>
<th>water</th>
<th>PG</th>
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<tbody>
<tr>
<td>OSAL</td>
<td>✓</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>DMI</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>water</td>
<td>×</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>PG</td>
<td>×</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
Table 4. The saturation level and thermodynamic activity of the test formulations containing 6% miltefosine (w/v).

<table>
<thead>
<tr>
<th>Formulations tested</th>
<th>Saturated? Yes/no (% saturation if known)</th>
<th>Thermodynamic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single solvent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>14%</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PG</td>
<td>5%</td>
<td>&lt;1</td>
</tr>
<tr>
<td>DMI</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>OSAL</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>Binary solvent system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG-DMI (1:1)</td>
<td>No</td>
<td>&lt;1</td>
</tr>
<tr>
<td>H₂O-DMI (1:1)</td>
<td>No</td>
<td>&lt;1</td>
</tr>
<tr>
<td>H₂O-PG (1:1)</td>
<td>No</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OSAL-DMI (1:1)</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>Ternary solvent system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSAL-DMI-PG (2:5:3)</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>H₂O-DMI-PG (3:4:3)</td>
<td>No</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
Table 5. Skin permeation parameters of miltefosine and the influence of solvents (H2O, DMI, OSAL and DMI-OSAL (1:1)). Each value represents the average±SD (n=4).

<table>
<thead>
<tr>
<th>Permeation parameters</th>
<th>6% miltefosine in H2O</th>
<th>DMI</th>
<th>OSAL</th>
<th>OSAL-DMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flux (µg/cm²/h)</td>
<td>3.1±2.4</td>
<td>16.6±5.6</td>
<td>15.6±12.4</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>Lag time (h)</td>
<td>16.2±2.1</td>
<td>18.0±4.7</td>
<td>21.0±0.5</td>
<td>5.2±4.1</td>
</tr>
<tr>
<td>Permeability coefficient (Kp) (cm/h)</td>
<td>7.1E-06±5.4E-06</td>
<td>2.7±1.3</td>
<td>0.6±0.4</td>
<td>0.02±0.01</td>
</tr>
</tbody>
</table>
Figure 1. Chemical structure and physicochemical properties of miltefosine.

Figure 2. Miscibility of ternary solvent mixtures. (black dots: miscible system; green dot: solvent system selected for in vitro permeation studies).

Figure 3. In vitro permeation assay using full-thickness BALB/c mouse skin in Franz diffusion cells. The cumulative amount of miltefosine that permeated per skin area expressed as a function of time (average±SD, n=4).

Figure 4. Skin disposition of miltefosine. Distribution of topically applied miltefosine formulations on the skin surface, extracted from the skin or that had permeated through the skin 48h after single-dose application. Data shown were obtained using full-thickness BALB/c mouse skin (average ±SD; n=4).

Figure 5. In vivo anti-leishmanial activity – lesion size. The in vivo activity of five formulations containing 6% (w/v) miltefosine in the non-healing cutaneous lesion model in BALB/c mice infected with L. major promastigotes. Lesions were treated with 50ul of formulation topically applied once daily for five days. The graph shows the progression of the average lesion size diameter per group as a function of time (n=5 except for vehicle control groups where n=3, average±SD). The rate of lesion size progression in the group receiving AmBisome is statistically significantly different from the other treatment groups (p<0.05, repeated measures ANOVA).

Figure 6. In vivo anti-leishmanial activity – parasite burden. The in vivo activity of five formulations containing 6% (w/v) miltefosine in the non-healing cutaneous lesion model in BALB/c mice infected with L. major promastigotes. Lesions were treated with 50ul of formulation topically applied once daily for five days. The graph shows the average parasite load per lesion per group two days after the last drug application (n=5 except for vehicle control groups where n=3, average±SD). The average parasite load in the marked groups (*) was statistically significant higher compared to the group receiving AmBisome (p < 0.05, One-Way ANOVA)