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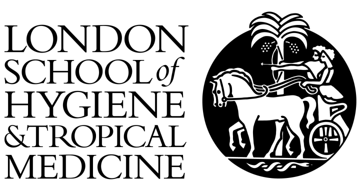

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1 **Informative title:** Topical formulations of miltefosine for cutaneous leishmaniasis in a BALB/c mouse
2 model

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11 **Abstract**

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

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

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

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

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

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

30 ***Introduction***

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

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

51 To permeate the *stratum corneum*, the main barrier to permeation for most topically applied drugs,
52 an active compound should ideally have a low molecular weight (< 500 g/mol) [12], less than three H-
53 bond donors [13], good solubility in the formulation vehicle and a partition coefficient between one
54 and three [14, 15]. The physico-chemical properties of miltefosine (Figure 1), are indicative of
55 potential skin permeation. Moreover, recent studies have shown enhanced permeation of drugs,
56 particularly drugs with high water solubility, through *Leishmania*-infected mouse skin [16] and a
57 topical miltefosine solution (Miltex®) has been used to treat superficial metastases of skin cancer,
58 indicating some drug permeation. . However, miltefosine is an amphiphilic and zwitterionic molecule
59 at skin pH (pH 5.5) containing both a positive and a negative charge (pKa ≈ 2) [17] and behaves as a
60 surfactant. The inherent aqueous solubility of such compounds challenges permeation through
61 lipophilic membranes such as stratum corneum.

62 Our aim therefore was to systematically investigate, for the first time, the potential of miltefosine as
63 a topical anti-leishmanial drug. We evaluated (i) the activity of miltefosine against a range of
64 intracellular *Leishmania* amastigotes *in vitro*, (ii) the permeation of miltefosine when applied to
65 BALB/c mouse skin in a range of solvents (propylene glycol (PG), water, dimethyl isosorbide (DMI) and
66 octyl salicylate (OSAL)) using Franz diffusion cells, (iii) the potential enhancement of *in vitro*
67 permeation using a combination of these solvents and (iv) the *in vivo* anti-leishmanial activity of the
68 optimal solvent systems upon topical administration in BALB/c mice. The solvents were chosen
69 because (i) they had a broad range of physicochemical properties, (ii) they have been reported to
70 enhance percutaneous drug delivery (Table 1), and (iii) they have been approved by the FDA as
71 inactive ingredient for drug products [18].

72 ***Materials and Methods***

73 **Materials**

74 Miltefosine was donated by Paladin Labs Inc (Montréal, Canada). The [^{14}C]-miltefosine (specific
75 activity: 36mCi/mmol, concentration: 900uCi/ml, radiochemical purity: 98.3%) was from Nycomed
76 Amersham Pharmacia (Buckinghamshire, UK). Schneider's insect medium, RPMI-1640 and M199
77 medium were obtained from Sigma Aldrich (Dorset, UK). Foetal calf serum was from Harlan
78 Laboratories (Derbyshire, UK) and heat-inactivated by exposure to 56°C for 30 minutes.

79 Propylene glycol (PG), dimethyl isosorbide ether (DMI), octyl salicylate (OSAL) and phosphate buffered
80 saline (PBS) tablets were from Sigma Aldrich (UK). The Franz diffusion cells, diameter of 0.90±0.03 cm
81 and mean receptor volume of 2.65±0.06 ml, were obtained from Soham Scientific (Fordham, UK). The
82 Optiphase™ supermix and Hionic Fluor™ liquid scintillation cocktails and Solvable™ were purchased
83 from Perkin Elmer (Coventry, UK). Liposomal amphotericin B (AmBisome®, Gilead Sciences, USA) and
84 amphotericin B deoxycholate (Fungizone®, ER Squibb, UK) were reconstituted according to
85 manufacturer's instructions.

86 ***Leishmania* parasites, cell culture and animals**

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

92 Female BALB/c mice (6-8 weeks old) were purchased from Charles River (Margate, UK) and housed in
93 a controlled environment of 55% relative humidity and 26°C. They were provided with tap water and

94 a standard laboratory diet. All animal experiments were approved by the Animal Welfare and Ethical
95 Review Board of the London School of Hygiene & Tropical Medicine and carried out under UK home office
96 licence (PPL 70/8207) according to the Animals (Scientific Procedures) Act 1986 and EU Directive
97 2010/63/EU.

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

99 Peritoneal exudate macrophages (PEMs) were harvested by lavage 24 hours after starch induction (2%
100 aq) from female CD-1 mice (LSHTM in house colony). The cells were seeded in 16-well Lab-Tek™ slides
101 in RPMI-1640 supplemented with 10% HiFCS at a density of 4×10^4 per well (100 µl). After 24 hours
102 incubation at 37°C and 5% CO_2/95% air mixture, the adhered PEMs were infected with stationary
103 phase promastigotes at a ratio of 3:1 (for *L. tropica* and *L. major*) or 5:1 (for *L. mexicana*, *L. aethiopica*
104 and *L. panamensis*) promastigotes and maintained at 34°C in a 5% CO_2/95% air mixture. 24 Hours later,
105 the infection was checked and the overlay replaced with medium-containing drug, in quadruplicate.
106 Final miltefosine concentrations were 30, 10, 3 & 1 µM. Amphotericin B (Fungizone®) was included as
107 control drug. After 72 hours incubation, all the slides were methanol-fixed and Giemsa-stained. Drug
108 activity was determined microscopically by counting the number of infected macrophages in drug-
109 treated cultures compared to untreated cultures. The EC50 and EC90 values were calculated by non-
110 linear sigmoidal curve fitting (variable slope) using Prism Software (GraphPad, UK).

111 **Exploration of topical drug delivery systems for miltefosine**

112 ***Drug solubility.*** The saturated solubility of miltefosine in PG, DMI, OSAL and water was determined as
113 described by [19]. Drug concentrations were determined by LC-MS (Finnigan LCQ, analytical
114 laboratory, LSHTM).
115 ***Solvent miscibility.*** In order to deliver the drug from a stable solvent system, the compatibility of
116 solvents was explored in binary and ternary phase miscibility studies. The appropriate volume and
117 ratio of solvents (v/v) were added into vials that were vortexed for two minutes and left to stand at
118 room temperature. After one hour, the samples were marked as immiscible (when phase separation
119 was visible) or miscible (when a homogeneous phase was seen). Ternary phase diagrams were drawn
120 using the OriginPro software (Northampton, UK)

121 ***Permeation and disposition of miltefosine in BALB/c mouse skin*****.** The test formulations contained 6%
122 (w/v) miltefosine (same as Miltex®) in the appropriate vehicle consisting of a single, binary or ternary
123 solvent mixture. After addition of miltefosine, the mixture was stirred overnight at 32°C. Before
124 application to the skin, the drug formulations were spiked with [^{14}C]-miltefosine to a final
125 concentration of 4 µCi/ml and vortexed for two minutes.

126 On the day of the permeation experiment, the mice were sacrificed and full-thickness dorsal skin was
127 removed, and cut to circular discs that were mounted between the donor and receptor compartments
128 of the Franz diffusion cells. The receptor fluid, PBS (pH 7.4), was sonicated and placed into the receptor
129 compartment together with a magnetic stirrer and the cells were incubated in a warm water bath
130 (32°C). After one hour, 500 µl of the test solution was applied to each donor compartment after which
131 200 µl of receptor phase was removed and replaced with fresh PBS at regular time intervals over a
132 duration of 48 hours.

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

142 After 48 hours, the permeation experiment was terminated and mass balance studies were conducted
143 as follows. The donor solution was transferred into a clean vial and any left overs were removed from
144 the skin by carefully wiping the surface with a cotton swab, followed by repeatedly pipetting with one
145 ml of a methanol:water (3:7 (v/v)) solution. This was repeated three times. To extract the miltefosine
146 absorbed in the cotton, one ml of a methanol:water (3:7 (v/v)) solution was added to the vial
147 containing the swab, and the mixture was left on a shaking plate for five hours, after which 100 µl of
148 the liquid was transferred to a flexible Microbeta 24-well plate. To quantify the amount of miltefosine,
149 400 µl of Hionic-fluor™ scintillation fluid was added and left to acclimatize before reading with a
150 Microbeta2 plate reader. For each formulation tested, a standard curve of 12 serial double dilutions
151 was included and each plate also contained three methanol:water blanks. The amount of miltefosine
152 in each sample was calculated from the miltefosine standard curve.

153 To determine the amount of miltefosine in the skin, the skin and one ml of Solvable™ were transferred
154 to a vial, incubated at 50°C and vortexed regularly until a homogenous mixture was obtained. A sample
155 of this homogenate (100 µl) was mixed with Hionic-fluor™ (300 µl) in a microbeta plate and analysed
156 using the Microbeta2 plate reader. Controls (in triplicate) included untreated skin spiked with a known
157 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 $2x10^7$ 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.*

190 *major* > *L. mexicana*. Amphotericin B, included as control drug, was highly active (in the nanomolar
191 range) against all species.

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

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

196 Miscibility of binary and ternary solvent mixtures are shown in Table 3 and Figure 2 respectively. PG,
197 water and DMI are miscible when combined in binary systems and hence the influence of
198 combinations of these solvents on the permeation of miltefosine were tested in permeation studies.
199 OSAL had limited miscibility with other solvents, being miscible with only DMI at a ratio of 1:1; this
200 binary mixture was also evaluated in permeation studies. To ensure miscibility throughout the
201 experiment, two ternary phase systems away from the miscible/immiscible boundary were selected.
202 These included OSAL-DMI-PG (2:5:3) and H_2O-DMI-PG (3:4:3). An OSAL-DMI-H_2O mixture was not
203 included due to limited miscibility.

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

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

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

216 The mass balance studies showed that only a very small fraction of the applied drug (< 4%) had
217 permeated through the skin from all formulations over 48 hours. Beyond 36 hours, an increase in
218 permeation was seen for most formulations in particular for the miltefosine in water formulation. This
219 was not unexpected as surfactants and particularly ionic surfactants such as miltefosine are known
220 skin irritants and have been reported to damage the skin [20-23], which would result in enhanced

221 permeation. Total drug recovery ranged from 76%-102% (Figure 4) with most of the applied drug
222 remaining on the skin surface. Miltefosine could not be detected in the skin when applied in PG, H_2O-
223 DMI, or the two ternary phase solvent systems. For the other six formulations, only small fractions of
224 the applied miltefosine ranging from 0.5-1.4% were found in the skin, with no significant difference
225 among the formulations tested ($p<0.05$).

226

227 ***In vivo* anti-leishmanial activity**

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

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

239 ***Discussion***

240 Miltefosine is a recommended oral treatment for both cutaneous and visceral leishmaniasis. We
241 confirmed the *in vitro* activity of miltefosine against a panel of species that cause CL with similar
242 activities to previously reported [24, 25]. Only the EC_{50} values for *L. mexicana* were higher, probably
243 due to the different strain used. Overall miltefosine was slightly less active against *L. mexicana* and *L.*
244 *major*, which was not unexpected as difference in intrinsic sensitivity to miltefosine across *Leishmania*
245 species is known [3, 24].

246 *In vitro* permeation assays showed limited permeation of miltefosine across full-thickness mouse skin
247 even when applied in different solvents or solvent mixtures. This was not unexpected as the stratum
248 corneum consists of dead cells surrounded by lipids, expecting to hinder the diffusion of hydrophilic
249 molecules such as miltefosine. Moreover, Miltex®, a topical solution of 6% miltefosine (w/v) in
250 propylene glycol ethers was indicated for cutaneous metastases of breast cancer with limited depth
251 [26] possibly due to the ability of miltefosine to permeate into the superficial layers of the skin where

252 the cancer cells are situated, while it is unable to reach the dermis where *Leishmania* amastigotes
253 reside inside macrophages.

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

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

280 Two clinical trials were conducted to evaluate the efficacy of topical Miltex® against CL (Bachmann P.,
281 unpublished data). One trial was conducted in Syria and included 16 patients with nodular CL who
282 applied the formulation twice daily. The other trial was conducted in Colombia where the 19 involved
283 patients received treatment once a day for 4 weeks [38]. Both trials of topically applied Miltex® were

284 unable to show efficacy against CL, even though oral administration has been shown to cure CL in
285 patients [4, 39].

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

291 Additionally, we observed that the lesion size results did not correlate with the parasite load per
292 lesion. A possible explanation could be (i) the skin irritation that exaggerated the lesion size readings
293 by enhancing the lesion size and (ii) the large variation in parasite burdens observed per group.
294 Moreover, severe skin irritation observed in the groups receiving miltefosine topically, required an
295 early halt to dosing. This was not surprising as surfactants such as miltefosine have been reported to
296 damage skin. Research has shown protein denaturation [20], swelling of the stratum corneum [21]
297 and lipid depletion and solubilisation in the stratum corneum [22]. Additionally, OSAL was reported to
298 be mildly irritating when applied to rabbit skin which could have caused further damage to the skin
299 [42].

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

304 ***Conclusion***

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

312

313 ***Conflict of Interest***

314 The authors state no conflict of interest.

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454

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

	Dimethyl isosorbide	**Octyl salicylate**	**Propylene glycol**	**Water**
			OH, OH	H, O, H
Mol. Wt. (g/mol)	174	250	76	18
Log $K_{O/W}$	0.07	5.97	-1.06	-1.38
Solubility parameter $(cal/cm^3)^{1/2}$	9.97	10.87	14.07	22.97
Density at 25°C (g/cm^3)	1.16	1.1	1.04	1.0
Penetration enhancement	[48]	[49-51]	[52, 53]	[54, 55]

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459 **Table 2.** *In vitro* anti-leishmanial activity as determined by microscopic counting of *Leishmania*
460 infected macrophages treated with miltefosine (30, 10, 3.3 and 1.1 uM; n= number of experiments).

Compound	n	Amphotericin B		Miltefosine	
		EC_{50} (µM) (95% CI)	EC_{90} (µM)	EC_{50} (µM) (95% CI)	EC_{90} (µM)
L. tropica	1	0.07 (0.06-0.07)	0.29	20.0 (17.4-23.0)	25.1
	2	0.08 (0.08-0.09)	0.30	9.4 (7.78-11.5)	-
L. major	1	0.12 (0.11-0.14)	0.22	44.9 (26.02-77.3)	163.1
	2	0.05 (0.04-0.06)	-	26.6 (21.30-33.2)	29.4
L. aethiopica	1	0.12 (0.11-0.12)	0.25	7.8 (6.2-9.8)	19.5
	2	0.11(0.10-0.12)	0.24	8.0 (7.26-8.7)	22.0
L. mexicana	1	0.43 (0.39-0.46)	1.10	31.0 (28.56-33.7)	38.0
	2	0.69 (0.55-0.69)	1.21	45.9 (36.61-57.5)	102.8
L. panamensis	1	0.14 (0.13-0.16)	0.29	20.0 (16.17-24.7)	151.3
	2	0.12 (0.09-0.14)	0.15	23.1 (20.41-26.2)	166.3

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464

465 **Table 3.** Miscibility of binary solvent mixtures (1:1 ratio, ✓ miscible; × immiscible).
466

	OSAL	DMI	water	PG
OSAL		✓	×	×
DMI	✓		✓	✓
water	×	✓		✓
PG	×	✓	✓	

467

468

469 **Table 4.** The saturation level and thermodynamic activity of the test formulations containing 6%
470 miltefosine (w/v).

Formulations tested	Saturated? Yes/no (% saturation if known)	Thermodynamic activity
Single solvent		
H_2O	14%	<1
PG	5%	<1
DMI	Yes	1
OSAL	Yes	1
Binary solvent system		
PG-DMI (1:1)	No	<1
H_2O-DMI (1:1)	No	<1
H_2O-PG (1:1)	No	<1
OSAL-DMI (1:1)	Yes	1
Ternary solvent system		
OSAL-DMI-PG (2:5:3)	Yes	1
H_2O-DMI-PG (3:4:3)	No	<1

471

472

473 **Table 5.** Skin permeation parameters of miltefosine and the influence of solvents (H2O, DMI, OSAL
474 and DMI-OSAL (1:1)). Each value represents the average±SD (n=4).

6% miltefosine in	H_2O	DMI	OSAL	OSAL-DMI
Permeation parameters				
Flux (μg/cm^2/h)	3.1±2.4	16.6±5.6	15.6±12.4	0.4±0.2
Lag time (h)	16.2±2.1	18.0±4.7	21.0±0.5	5.2±4.1
Permability coefficient (K_p) (cm/h)	7.1E-06±5.4E-06	2.7±1.3	0.6±0.4	0.02±0.01

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479 **Figure 1. Chemical structure and physicochemical properties of miltefosine.**

480

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

483

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

487

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

491

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

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