ORIGINAL ARTICLE

Efficacy of topical Miltefosine formulations in an experimental model of cutaneous leishmaniasis

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Abstract

Cutaneous leishmaniasis (CL) is a neglected tropical disease endemic in ~ 90 countries, with an increasing incidence. Presently available pharmacotherapy implies the systemic administration of moderately/very toxic drugs. Miltefosine (Milt) is the only FDA-approved drug to treat CL via the oral route (Impavido®). It produces side effects; in particular, teratogenic effects are of concern. A topical treatment would have the great advantage of minimising the systemic circulation of the drug, preventing side effects. We prepared dispersions containing Milt and liposomes of different compositions to enhance/ modulate trans-epidermal penetration and evaluated in vitro and in vivo efficacy and toxicity, in vitro release rate of the drug and particles size stability with time. Treatments were topically administered to BALB/c mice infected with *Leishmania* (*Leishmania*) *amazonensis*. The dispersions containing 0.5% Milt eliminated 99% of the parasites and cured the lesions with a complete re-epithelisation, no visible scar and re-growth of hair. Fluid liposomes decreased the time to heal the lesion and the time needed to eliminate viable amastigotes from the lesion site. Relapse of the infection was not found 1 month after treatment in any case. Ultraflexible liposomes on the other hand had no significant in vitro effect but decreased in vivo efficacy. A topical Milt formulation including fluid liposomes seems a promising treatment against CL.

Keywords Cutaneous leishmaniasis · Miltefosine · Liposomes · Penetration enhancers · Topical treatment

Introduction

Leishmaniasis is an orphan tropical disease caused by *Leishmania* parasites, transmitted by hematophagous vectors of the genus *Lutzomyia* in the New World and *Phlebotomus* in the Old World. The progress of leishmaniasis depends on the parasite species and the immune state of the host. Cutaneous leishmaniasis (CL) is the most prevalent form in all the endemic countries, and its incidence is increasing worldwide [1], with 0.7–1.2 million cases per year [2].

Formerly a disease typical of rural, isolated areas, CL is appearing more frequently in periurban areas [3, 4]. There are more than 20 species of *Leishmania*, all of them with different sensitivities to each pharmacological treatment. In the case of patients with a competent immune system, CL resolves spontaneously after few weeks/months, leaving a scar. It can however complicate into the muco-cutaneous or even the visceral form, especially in immunocompromised patients [5].

Current pharmacotherapy involves toxic drugs administrated intravenously, intramuscularly or orally. Systemic toxicity of the first-line treatment with pentavalent antimonials makes it sometimes mandatory for the patient to be either hospitalised or frequently monitored for renal/hepatic function [6], increasing the costs of the treatment [7–9] and often forcing the patient to move to a city with laboratory/hospital infrastructure. This produces extra costs in transportation, food and lodging to the patient and his family, while in the meantime he/she cannot work [10].

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Available local treatments (intra-lesional injections with antimonials, cryotherapy, thermotherapy) require highly trained medical personnel, are painful and/or require special equipment [11]. Of note, thermotherapy, intralesional injections and oral Miltefosine capsules are currently not available in Argentina, where the disease is endemic and affects at least 400 people per year [12].

The development of topical formulations of different drugs to treat CL is presently a subject of very active research since they could minimise or prevent the systemic circulation of the drug, which would mean fewer side effects [13], solving many of the above problems. A topical treatment would not produce pain enhancing patient compliance; it would avoid gastrointestinal/renal/hepatic/neurological side effects, thus allowing its use in regions where no hospital infrastructure is available; it could be used in children or even pregnant women; it could be self-administered by the patient. If it allowed a fast and ordered healing, it would minimise the probability of secondary bacterial infection (the most common complication of CL) and it would prevent the scar that usually remains in untreated lesions that heal spontaneously [14]. In addition, the production of a topical treatment has a lower cost of production than a parenteral treatment. This should allow for a lower market price of the formulation.

A topical formulation including amphotericin B 3% (Anfoleish) has recently failed in a phase II clinical trial due to its low efficacy [15]. Topical paromomycin has been reported as effective against some species of *Leishmania*, but the information on effectivity against American species is contradictory and scarce. Besides, the treatment needs to be long, local reactions occur in all cases and the formulations are not commercially available [16–18].

Hernandez and co-authors [19] have reported the in vitro activity of Miltefosine (Milt) incorporated in liposomes being more active against *L. (V.) braziliensis* than *L. (V.) panamensis*. Momeni and co-authors [20] reported a series of liposomal formulations with different drugs against cutaneous leishmaniasis induced by *L. (L.) major* in vivo. The formulations, applied as injections in the sites of the lesions, gave the best results when they contained Milt, although none produced a 100% cure or complete elimination of parasites in the lesions.

Miltefosine is an alkyl-phosphoryl-choline with a high solubility in water (> 2.5 mg/ml). It was originally developed as an anti-cancer drug and later found to be effective against various forms of *Leishmania*, including *L. (L.) amazonensis* [21–25]. Milt was first approved for use in human visceral leishmaniasis in India in 2002. In 2014, the FDA-approved Milt oral capsules to treat human visceral, cutaneous and muco-cutaneous leishmaniasis in patients older than 12 years of age [26].

Oral Milt still produces side effects (mainly gastrointestinal), and the record of teratogenicity in pre-clinical trials limits its use in children and fertile/pregnant women [27]. Importantly, children comprise between 7 and 70% of all CL patients, depending on the region [3, 28–30]. Fertile women must use a contraception that should not be orally administered due to the frequent vomiting and diarrhoea produced by oral Milt, further complicating treatment. In vivo, a commercial form of Milt (Miltenox®) administered orally showed efficacy against *L. (L.) amazonensis* but damage in the liver and spleen was found [31].

A topical formulation (Miltex®) containing 6% Milt is commercially available and is indicated for the treatment of breast cancer lesions. Schmidt-Ott and collaborators tested this formulation in mice infected with *L. (L.) major* and *L. (L.) mexicana* and found an important reduction in parasite burden against *L. (L.) major* but relapse in animals infected with *L. (L.) mexicana* [32]. On the other hand, Van Bocxlaer and collaborators topically applied formulations of Milt 6% in different solvents (including water) to mice infected with *L. (L.) major* and found irritation and low anti-*Leishmania* activity [33]. The information on the therapeutic value of topical formulations of Milt 6% is therefore contradictory or negative.

In the topical treatment of CL, it is of the outmost importance that the active compounds can penetrate the skin and reach the amastigotes, which are localised in the dermis below the ulcer. Since the literature indicates that Milt penetrates in very small amounts through the skin [33], the use of a penetration enhancer is probably necessary in order to increase the drug's concentration near the parasites.

Liposomes in a topical formulation modulate the penetration of drugs through the skin [34]. Ultraflexible/fluid liposomes enhance the penetration of hydrophilic drugs of high molecular weight in healthy or damaged skin [35]. Ultraflexible liposomes are prepared by the addition of a detergent like Na Cholate, which increases the flexibility of the liposomal membrane, enhances their penetration through the skin and aids in the stability of the formulation because of the negative charge of the detergent [36–39]. In open lesions, it has been shown that different active compounds have an enhanced ability to improve wound healing when incorporated in liposomes [35, 40, 41].

With this scenario, the goal of this work was to find a topical formulation containing Milt and liposomes that is able to give a good therapeutic result in vivo. We designed fluid and ultrafluid liposomes with two different amounts of incorporated Milt and studied in vitro efficacy, cytotoxicity and irritation. Topical in vivo efficacy was evaluated in an animal model of CL caused by *L. (L.) amazonensis* and was found to be extremely good for two of the preparations. To better understand the impact of different components on efficacy, we performed a physicochemical characterisation of the best performing formulations.

Materials and methods

Ethics

Animal experimental protocols were approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio from Universidad Nacional de Salta (Res. CD No. 745-17). Procedures followed the Guide for the Care and Use of Laboratory Animals of NIH and complied with the ARRIVE guidelines and with Argentinian Law 14346 on Animal Protection.

Preparation of liposomes

We used two different kinds of liposomes. Liposomal membranes composed of unsaturated phospholipids are fluid at temperatures above 10 °C [42]. Ultraflexible liposomes result from the mixture of unsaturated phospholipids with a single-chain surfactant/edge activator [43]. We therefore used unsaturated phospholipids to produce fluid liposomes (FLs) and a mixture of unsaturated phospholipids and an edge activator, in this case Na cholate (NaChol), to prepare ultraflexible liposomes (UFLs).

Liposomes were prepared by the thin-film method [44]. Briefly, an appropriate amount of unsaturated phospholipids (PLs) (Avanti Polar Lipids, Alabama, USA) (for fluid liposomes (FLs)) or unsaturated phospholipids (PLs) + NaChol (6:1 w:w phospholipid:NaChol) (Sigma Aldrich, St. Louis, USA) (for ultra-fluid liposomes, UFL) were dissolved and mixed in methanol:chloroform (Emsure, Germany) 1:2 v/v. The solvent was evaporated with N_2 under constant rotation in a glass vial, and then, 2 h of vacuum was applied to eliminate any residues of solvent. The resulting lipidic thin film was hydrated by adding HEPES buffer 30 mM, pH 7.4, at room temperature up to a final lipid concentration of 50 mg/ml. Multi-lamellar liposomal dispersions thus produced were extruded 17 times through 100 nm pore polycarbonate membranes using a 1 ml extruder (Avanti Polar Lipids, Inc., AL, USA) at room temperature to obtain a homogeneous dispersion of 100 ± 30 nm uni-lamellar liposomes.

Preparation of Miltefosine and lipid dispersions

For in vitro evaluation of cytotoxicity, irritation and efficacy

For treatments with pure Milt, n-hexadecylphosphocholine (Avanti Polar Lipids, Alabama, USA) solution was prepared at 1 mg/ml in HEPES buffer (AppliChem, Germany) (30 mM, pH 7.4 30 mM, pH 7.4) and filtered with 0.22 μm pore sterile carbonate membranes under the hood to ensure sterility. For treatments with dispersions of Milt-FL and Milt-UFL, unilamellar liposomes in HEPES buffer were prepared by the thin film method followed by extrusion as described above (see "Preparation of liposomes"). The extrusion was used also to sterilise the samples, by using sterile 0.1 μm pore carbonate membranes in a sterile extruder under the hood. Appropriate amounts of Milt, FL and UFL dispersions in HEPES buffer were mixed under magnetic stirring. Concentrations for the Milt-FL dispersions in buffer were 30 mM for Milt and 220 mM for PL (PL/Milt molar relation = 7.33) or 30 mM for Milt and 7 mM for PL (PL/Milt molar relation = 0.23). Concentrations for the Milt-UFL in buffer were Milt:PL:NaChol 30:220:77 mM (PL/Milt molar relation = 7.33; PL/NaChol molar relation = 2.86). All subsequent dilutions of each treatment were made with supplemented cell culture medium under sterile conditions, before incubation with cells.

For in vivo efficacy

In order to facilitate topical application of the dispersions, a Carbomer 934-P NF (a carboxypolymethylene polymer) (Saporitti®, Bs. As., Argentina) hydrogel was prepared by hydration with Milli-Q water at a concentration of 1% w/v and thoroughly mixed with either a Miltefosine (Milt) solution or both the Milt solution and one of two liposomal dispersions. The final Milt concentrations in the gels were 0.015 and 0.5% w/v. For gels containing only Milt, the drug was dissolved in HEPES buffer 30 mM, pH 7.4, and this solution was mixed, at 1:1 volume ratio, with Carbomer 934-P NF 1% w/v, pH 6, at room temperature. For gels containing liposomal dispersions, 5% of the HEPES buffer used to prepare the gel was replaced by the liposomal dispersion. The formulations were stored at 4 °C and visually inspected for homogeneity and stability.

In vitro evaluation of irritation, cytotoxicity and efficacy

For cytotoxicity assays, RAW 264.7 (murine) macrophages were cultured in RPMI 1640 medium supplemented with FBS 10%, 100 U/ml penicillin and 50 mg/ml streptomycin (P-S). Macrophages (3×10^4) were seeded in a 96 multi-well plate with 100 μl of supplemented medium without phenol red and treated by adding 100 μl of the samples diluted in medium at different drug/liposome concentrations. The cultures were then incubated at 37 °C, 5% CO_2 during 48 h. The viability was determined by MTT assay. Briefly, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37 °C; the supernatant was discarded, and formazan crystals were dissolved with 200 μl of DMSO. Absorbance was determined by an ELISA detector at 560 nm, and macrophages inhibition (%) was calculated as 100 − sample absorbance/control absorbance × 100.

For irritation assays, L929 (murine) fibroblasts were cultured in DMEM:F12 medium (Gibco, USA) supplemented with FBS 10%, 100 U/ml penicillin and 50 mg/ml streptomycin at 37 °C, 5% CO_2. Cells (1×10^4) were seeded in 96-wells chambers in 100 µl of culture medium without phenol red and allowed to attach for 24 h. The culture medium was removed and replaced by 100 µl of each treatment dissolved in culture medium. After 24-h incubation at 37 °C, 5% CO_2, the viability was determined by MTT assay as previously described. Fibroblast viability (%) was calculated as sample absorbance/control absorbance × 100.

For efficacy assays, RAW 264.7 macrophages (1×10^4) were seeded in 8-well plates with 200 µl of supplemented medium and incubated at 37 °C for 24 h to allow cell adherence. L. (L.) amazonensis (MHOM/BR73/M2269) promastigotes were added in a ratio of 20 parasites per macrophage in 50 µl of medium and incubated at 33 °C for 5 h. Wells were washed with sterile proline balanced salts solution (PBSS) to eliminate nonadhered parasites and incubated in supplemented medium at 33 °C for 48 h to ensure promastigote conversion to amastigotes. Medium was replaced by treatments diluted in fresh medium, and cells were incubated for an extra 48 h at 37 °C, 5% CO_2. Medium was discarded and cells were fixed and dyed with Diff Quick kit (Biopur S.R.L., Rosario, Argentina), which stains both macrophages and amastigotes with purple in the nucleus and light blue in the cytoplasm. The number of infected macrophages and parasites per cell were determined in 300 macrophages by direct counting under a Zeiss optical microscope at 100x. Infection index (%) was calculated as percentage of infected macrophages × mean number of parasites per cell. Finally, parasite inhibition (%) was calculated as 100 − sample infection index/control infection index × 100 [21].

In vivo efficacy in a murine model of cutaneous leishmaniasis

A scheme of the experiments is shown in Fig. 1a. BALB/c mice [45] were intradermally injected with $1–2 \times 10^7$ stationary phase L. (L.) amazonensis (MHOM/BR73/M2269) promastigotes (50 µl) in the rump above the tail. Five weeks after infection, when the ulcer was visible, the treatments were started. Two experiments were carried out. Mice were randomly allocated in groups of five as follows: control group (without treatment), Milt group, Milt-UFL and Milt-FL group. The formulations (100 µl) containing 0.015% and 0.5% of Milt were topically administered covering both the border and the centre of the ulcer, once or twice a day, 5 days a week, during 21 days. After the administration of the treatments, the mice were observed for about 30 min to verify the absorption of the gel during this period and that the animals did not lick their wounds. At the end point of experiments, animals were euthanised with a ketamine/xilacine overdose. The efficacy of the treatments was assessed by the following means:

Lesion areas. Each lesion was measured weekly with a digital calliper during treatments. The area (mm^2) was estimated as $(d_1/2) \times (d_2/2) \times \pi$, where d_1 is the larger diameter and d_2 is the smaller diameter.
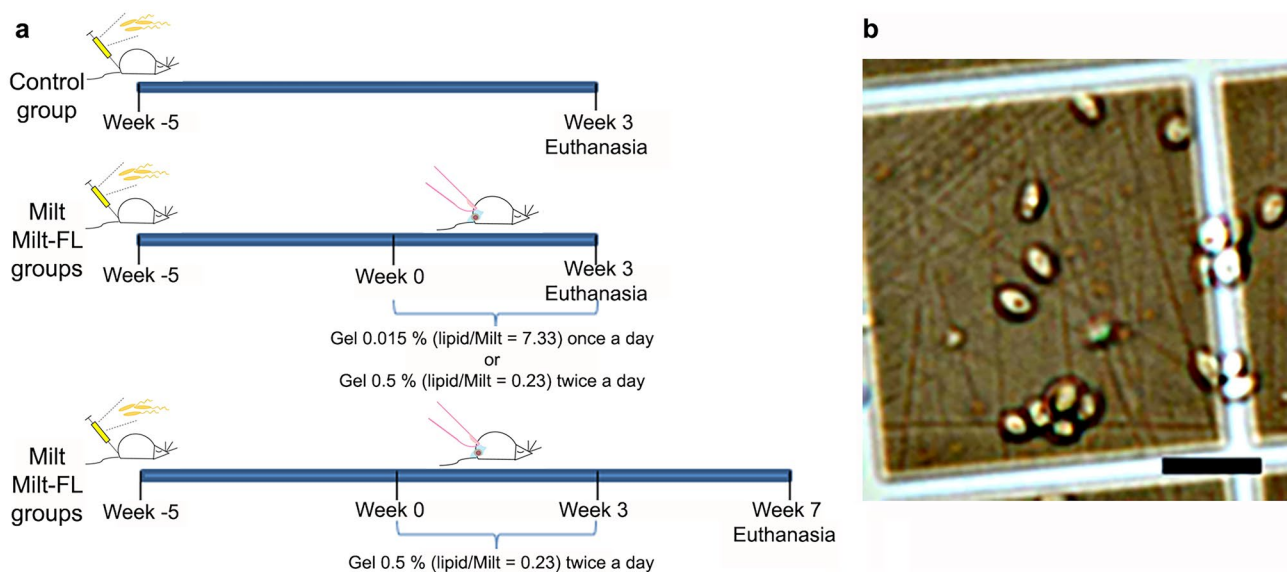

Fig. 1 **a** Scheme describing in vivo efficacy assays timeline. **b** Optical microscopy image of L. (L.) amazonensis amastigotes obtained from mice lesions at the end of treatment. The objective used was a 40x. Scale bar, 5 microns

Histology. Half of each lesion was immersed in 25 ml of 10% formol buffer. After 48 h, the lesions were embedded in paraffin, cut transversally and dyed with haematoxylin–eosin.

Parasitic burden. Skin tissue samples (ulcers or cicatrised ulcers) were aseptically removed and weighed. To obtain the amastigotes from tissue, we followed the protocol described by Lima [46]. Briefly, skin tissue samples were homogenised in 25 ml of sterile proline balanced salts solution (PBSS) containing 100 U/ml penicillin and 50 mg/l streptomycin (P-S); the amastigotes were separated through mechanical tissue disruption using a glass grinder. The homogenates were seated for ≥ 1 min on ice to allow chunk to drop to the bottom of the tube. The concentration of parasites (amastigotes/ml) of each homogenate was determined by direct counting under a Zeiss optical microscope at 40x in a Neubauer chamber [47, 48] (Fig. 1b). It is important to mention that *L.* (*L.*) *amazonensis* amastigotes are easily identified by their size (2 µl) and oval shape without flagellum, as described by Chang and collaborators [49]. The total number of parasites in each homogenate was calculated as amastigotes/ml × 25 ml; then, the results were adjusted per milligram of tissue [50]. The percentage of parasite inhibition with regard to controls was calculated as (average total number of amastigotes/mg of tissue in treated group) × 100/(average total number of amastigotes/mg of tissue in control group). Previously, we validated this method for measuring parasitic burden from tissue using parasites of *L.* (*L.*) *amazonensis* (MHOM/BR73/M2269) expressing tandem dimeric tomato red fluorescent protein. The relationship between the number of recovered amastigotes counted under the optic microscope with the fluorescence intensity measured in the same samples was a straight line. The regression analysis shows a slope value of 1 and an $R^2 = 0.98$ (Barroso, PA; Pérez Brandán, C; Portelli, S; Bracamonte, E; Hoyos, C; Basombrío, MA; Marco, JD. Development of *Leishmania* (*Leishmania*) *amazonensis* expressing fluorescent tomato red protein for in vitro and in vivo screening of anti-*Leishmania* drugs. Worldleish 6. Toledo, Spain. May 16–20, 2017). We are thus confident that both assays are truly measuring the number of amastigotes obtained from tissue.

Amastigote viability. A sample of each homogenate (1 ml) was cultured in Difco blood agar (USMARU) medium containing 20% of defibrinated rabbit blood plus P-S [51, 52] since it is the most appropriate culture medium for parasite isolation. In order to determine if visceralisation of infection occurred in infected mice, samples of spleens were also cultured in USMARU medium plus P-S. The cultures were examined for the presence of *Leishmania* promastigotes after 1 week. Negative cultures were examined during 1 month.

***Leishmania*-specific antibodies.** IgG1 and IgG2a were measured by ELISA in serum (samples taken at the end of the treatment or 1 month later). Plates were sensitised with *L.* (*L.*) *amazonensis* antigen (1.75 mg/l), blocked with PBS-Tween + 5% milk and treated with mouse serum samples (1/50). IgG1 and IgG2a (BD Pharmigen Biotin Rat Anti Mouse IgG1 and IgG2a, BD Biosciences) and Peroxidase Avidine were added in 1/3000 and 1/5500 dilutions respectively. The plate was revealed with 1 ml of 10× phosphate citrate buffer, 1 ml of TMB in DMSO, 8 ml of bi-distilled water and 10 µl of peroxide hydrogen. The reaction was stopped with H_2SO_4 (0.5 N) and read at 405 nm in a plate reader TECAN Infinite 200 PRO.

Hepatic enzymes. GOT (AST) and GPT (ALT) enzymes were determined by aspartate aminotransferase and alanine aminotransferase acc. to IFCC without pyridoxal phosphate activation according to the guidelines of the manufacturer. A Roche/Hitachi Cobas® c 701/702 analyser was used to quantify the results.

Statistical analysis. Statistical analyses were performed with a nonparametric Mann–Whitney test at different *p* values. The software GraphPad (v.6) was used. Results are informed as mean and standard error.

Electron microscopy

Aqueous and hydrogel lipid dispersions prepared as described for in vivo efficacy experiments were diluted to 2 mg/ml (lipid concentration) and characterised by TEM using a LEO 906-E Zeiss at 80 kV. One percent phosphotungstic acid was used as negative staining solution. Two hundred mesh nickel electron microscope grids coated with formvar were used as support.

Dynamic light scattering

Particle hydrodynamic diameters were measured on the same day of preparation and once a week for 45 days by dynamic light scattering (ZetasizerNano ZS, Malvern, UK). Two hundred microliters of the gel samples prepared as described for in vivo efficacy studies were diluted in 4 ml of HEPES buffer. Measurements were done at 23 ± 1 °C in triplicate.

Thin-layer chromatography

Change upon storage of lipids and Milt in the hydrogels was evaluated by thin-layer chromatography (TLC) following the method of Skipski et al. [53, 54]. Gels containing Milt at 0.5% and FL, prepared as described for in vivo assays, were analysed immediately upon preparation and after 2 and 9 months of storage at 4 °C. One hundred and fifty microlitres of gel samples were lyophilised and re-suspended in 750 µl of chloroform. Up to 30 µl of the re-suspended material was spotted onto TLC alumina plates with a micro syringe (TLC aluminum sheets, silica gel layer, ALUGRAM

Xtra SIL G UV254, 20 × 20 cm, Macherey–Nagel, Germany). The chromatograms were developed for 2 h with methanol:chloroform 2:1 v:v as the mobile phase in a glass chamber. The plates were then air-dried at room temperature for 4 min and heated to 170 °C for 10 min. To visualise the spots, the plates were submerged for 20 s in 10% $CuSO_4$ in 8% H_3PO_4, dried by placing them vertically on filter paper for 30 s and taken to an oven at 170 °C for 16 min. The resulting spots were scanned or photographed, and the images analysed with ImageJ. The experiments were performed in triplicate.

To quantify Milt, freshly prepared solutions of Milt in chloroform were used as concentration standards. A minimum of 2 mg of Milt was measured with an analytical electronic balance and used to prepare a stock solution in chloroform. The necessary reference solutions were prepared by dilution of this stock and kept for no more than 3 weeks at − 20 °C. In our hands, this method proved to give quantitative, linear results in the range 2–100 nmol of Milt. The minimum amount of material that could be detected was 1 nmol of Milt.

Miltefosine in vitro release kinetics

Milt release was quantified in liposomal samples (PL/Milt molar relation 0.23) and in pure Milt solutions at Milt concentrations of 2.7% w/v. This high amount of drug was necessary to detect it in the receptor compartment by TLC; however, *sink* conditions were ensured (Milt concentrations penetrating in the receiver were not larger than 10% of the saturation solubility concentration of the drug). Milt release was evaluated in vertical Franz cells with 0.2 cm² exposed area. The donor compartment was filled with Milt (4 mg) in the presence/absence of extruded liposomes (1.72 mg of phospholipid) suspended in 150 µl of HEPES buffer, pH 7.4. The receptor compartment was filled with 5 ml of Milli-Q water and the whole setup was maintained at 35 ± 1 °C under agitation for 24 h. Polycarbonate membranes with 100 nm pores (Avanti Polar Lipids, Inc., AL, USA) were used to separate donor/receptor compartments. At fixed times, 150 µl of sample were taken from the receptor medium and replaced by fresh water at 35 ± 1 °C. Samples were frozen to − 80 °C, lyophilised, dissolved in 50 µl of chloroform and quantified by TLC as previously described. The lyophilisation/re-dissolution procedure recovered 100% of original lipid/Milt. To determine Milt concentration, a five-point concentration curve of Milt was run alongside each set of samples in the same plate. Release kinetics was studied by fitting the release patterns to zero-order and Higuchi models [55].

Results

In vitro evaluation of irritation, cytotoxicity and efficacy

We designed the following liposomes:

- Ultrafluid liposomes composed of PL:NaChol:Milt, with PL/NaChol molar ratio of 2.86 and PL/Milt molar ratio of 7.33.
- Fluid liposomes composed of PL:Milt, with PL/Milt molar ratios of 7.33 and 0.23.

We then proceeded to evaluate their performance in vitro. For clarity, we have separated the description of these results in three sections.

Effect of fluid liposomes on Miltefosine cytotoxicity

Cytotoxicity of dispersions was assayed against murine macrophages. Two different mixtures of Milt and fluid liposomes were studied: PL/Milt molar relation of 7.33, and PL/Milt molar relation of 0.23. While keeping these relationships constant, Milt concentration was increased from 2.3 to 36 µM and PL concentration varied accordingly. Pure Milt was cytotoxic (38% cell death) against macrophages at concentrations higher than 9 µM (Fig. 2a, black bars). Empty fluid liposomes were not toxic against macrophages at any concentration assayed (Fig. 2c, red bars). When a small amount of PL was added (PL/Milt molar relation of 0.23), the cytotoxicity of Milt decreased slightly (Fig. 2c, red bars with diagonal stripes). When a higher PL/Milt relationship was used (PL/Milt molar relation of 7.33), the cytotoxicity of Milt was greatly inhibited (Fig. 2a, green bars with diagonal stripes). At 9 µM of Milt for example, the cytotoxicity on macrophages was lowered from 38 to 10%.

Effect of fluid liposomes on Miltefosine parasitic inhibition

In order to assess efficacy against *Leishmania* amastigotes, assays were carried out at Milt concentrations below 9 µM because of the high cytotoxicity against macrophages at higher concentrations. Milt showed a dose-dependent effect on parasitic inhibition against *L. (L.) amazonensis* amastigotes. The inhibition was high (approx. 40%) even at the lowest concentration assayed of 2.3 µM. At 9 µM, Milt parasitic inhibition was 90% (Fig. 2b, black bars). Surprisingly, empty fluid liposomes (FLs) produced high parasitic inhibitions of 20–40% in the range of concentrations assayed (0.6–2.3 µM phospholipid) (Fig. 2d, red bars). Also, when liposomes were added to
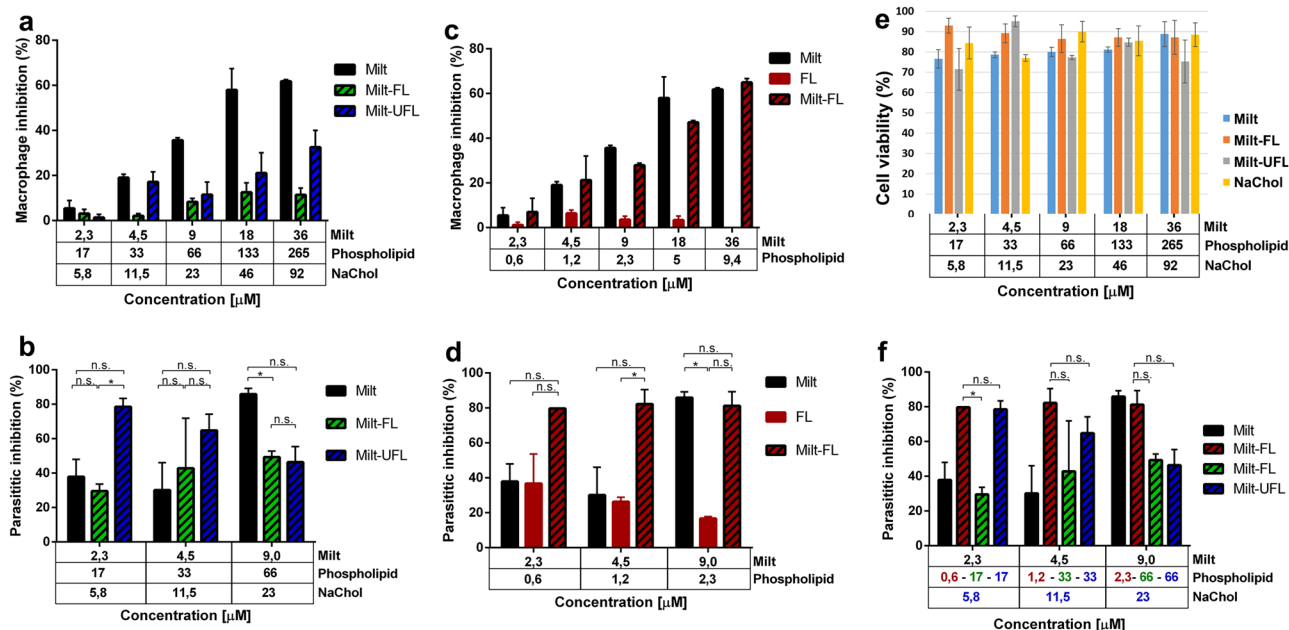
Fig. 2 In vitro cytotoxicity, irritation and efficacy data of Miltefosine (Milt), liposomes and liposomes + Milt. **a**, **c** Toxicity of Milt and liposomes against macrophages. Cytotoxicity data of increasing concentrations of Milt, fluid liposomes + Milt (Milt-FL) and ultra-fluid liposomes + Milt (Milt-UFL) (lipid/drug molar ratio 7.33) **a**. Cytotoxicity of Milt, fluid liposomes (drug free) (FL) and fluid liposomes + Milt (Milt-FL) (lipid/drug molar ratio 0.23) **c**. **e** In vitro irritation assay. Percentage viability of murine fibroblasts in the presence of increasing concentrations of Milt, fluid liposomes + Milt, ultra-fluid liposomes + Milt (lipid/drug molar ratio 7.33) and NaCho-late. **b**, **d**, **f** Efficacy of Milt and liposomes against *Leishmania* amastigotes in infected macrophages. Efficacy of increasing concentrations of Milt, fluid liposomes + Milt and ultra-fluid liposomes + Milt (lipid/drug molar ratio 7.33) **b**. Efficacy of increasing concentrations of Milt, fluid liposomes (drug free) and fluid liposomes + Milt (lipid/drug molar ratio 0.23) **d**. Comparison of the efficacy against amastigotes of Milt, fluid liposomes + Milt (lipid/drug molar ratio 0.23), fluid liposomes + Milt (lipid/drug molar ratio 7.33) and ultra-fluid liposomes + Milt (lipid/drug molar ratio 7.33) **f**. Experiments were performed in triplicate. Results are shown as mean ± EE

Milt, a small proportional amount of phospholipids (PL/Milt molar relation = 0.23) produced a notable increase in the efficacy against amastigotes, taking the parasitic inhibition to 85–90% at all assayed concentrations of Milt (Fig. 2d, red bars with diagonal stripes). On the other hand, a higher proportional amount of phospholipids (PL/Milt molar relation = 7.33) decreased the parasitic inhibition compared to the low proportional amount of phospholipids assayed (Fig. 2b, f, green bars with diagonal stripes). The coexistence of Milt and phospholipids at high PL/Milt molar relation did not change Milt efficacy, except at the highest Milt concentration assayed (Fig. 2b, f, green bars with diagonal stripes).

Effect of sodium cholate

Cytotoxicity was assayed against murine macrophages, while fibroblasts were used as indicators of possible skin irritation. Liposomes containing Milt, PL and NaChol (ultraflexible liposomes (UFLs)) diminished cytotoxicity compared to Milt alone, but the protective effect was weaker than in the case of fluid liposomes (Fig. 2a, blue striped bars).

The addition of NaChol to liposomes at PL/Milt molar relation of 0.23 increased the parasitic inhibition at low Milt concentrations compared to Milt alone (Fig. 1b, f, striped blue vs black bars), but this difference was smaller at higher Milt concentrations, and at the highest assayed concentration the parasitic inhibition of Milt alone was higher than that of Milt either in FL or in UFL liposomes.

Regarding irritation, concentration ranges assayed were 2.25–36 μM for Milt and 5.8–92.2 μM for NaChol. In mixtures, PL and NaChol concentration varied following Milt concentration according to fixed molar relations: PL/Milt molar relation was 7.33, and PL/NaChol molar relation was 2.86. Results show that neither NaChol, Milt, Milt-FL or Milt-UFL dispersions were cytotoxic against fibroblasts in the assayed range of concentrations (cells viability remained above 72%) (Fig. 2e).

In vivo efficacy assays

Throughout this section and for brevity, we will not always add the word "gel" or "hydrogel" to describe the formulations tested. Nevertheless, all in vivo experiments

were performed with the active ingredients (Milt, FL, UFL either alone or in combination) included in a Carbomer hydrogel.

A first approximation to the treatment of CL with Milt was performed with Milt at 0.015% in one dose per day during 3 weeks. We used either a gel containing only Milt or a gel containing the same concentration of Milt plus UFL. Even though no significant differences were observed in lesion size between treated mice and control group at the end of the first experiment, Milt could arrest the increase of lesion size in the last week of treatment (Fig. 3a) and diminished the parasite load by 68% compared with the control group (Fig. 3b). However, in mice treated with Milt-UFL the inhibition of parasite load was much lower (7.5%) (Fig. 3b). The amastigotes recovered from the ulcers of Milt and Milt-UFL groups showed a good vitality since they were able to transform into promastigotes in USMARU medium.

In a second experiment, the concentration of drug was increased to 0.5% and two doses per day during 3 weeks were applied in order to improve the parasitic inhibition. On the other hand, NaChol was eliminated from liposomes because of the above results and because, in simultaneous experiments with a different drug, we also found that the presence of NaChol produced a decrease of therapeutic efficacy.

In this second experiment therefore, the liposomes used were fluid but not ultraflexible. Both treatments (gels containing Milt and Milt-FL) decreased the lesion size compared with the control group (Fig. 4a). Mice treated with Milt-FL and Milt showed a parasite inhibition of 99.7 and 99.8%, respectively (Fig. 4b). At day 21 of treatment, we observed clinical cure (ulcer closure) in all the animals;

however, a small cicatrix remained in 7 of 10 ulcers treated with Milt and 4 of 10 in lesions treated with Milt-FL. Also, more hair had grown in the lesion site of animals treated with Milt-FL than in animals treated with Milt (see photographs in Fig. 4).

Notice the high therapeutic efficacy of Milt at 0.5% (Fig. 4a, light grey bars). The lesion practically disappears, and the parasitic inhibition is very high (99.8%) (Fig. 4b). In these mice, however, the culture of the tissue in USMARU medium was positive, indicating that immediately after the end of treatment, the little number of parasites left in the skin were still viable (able to transform into promastigotes). The addition of fluid liposomes increased the efficacy of Milt, to the point that no viable parasites were found in the lesion region immediately after the end of treatment (culture in USMARU was negative, Fig. 4b). The statistical comparison of lesion sizes with the control group shows highly significant differences ($p < 0.004$) for Milt and Milt-FL at every tested point. Also, at every point during treatment, lesions were smaller in the Milt-FL group compared to the Milt group, although the differences are not statistically significant ($p < 0.016$).

To study reactivation (the appearance of a lesion within or at the border of a previously healed lesion [56]), two groups of animals, one treated with Milt and one with Milt-FL, were kept alive for 1 month after finishing the treatment. We found parasites in the healed skin of mice from the Milt group but they were not able to transform into promastigotes (Fig. 5a). No parasites were observed in skin samples of the Milt-FL group. In addition, no visceralisation was observed in any of these animals since spleen cultures were negative.
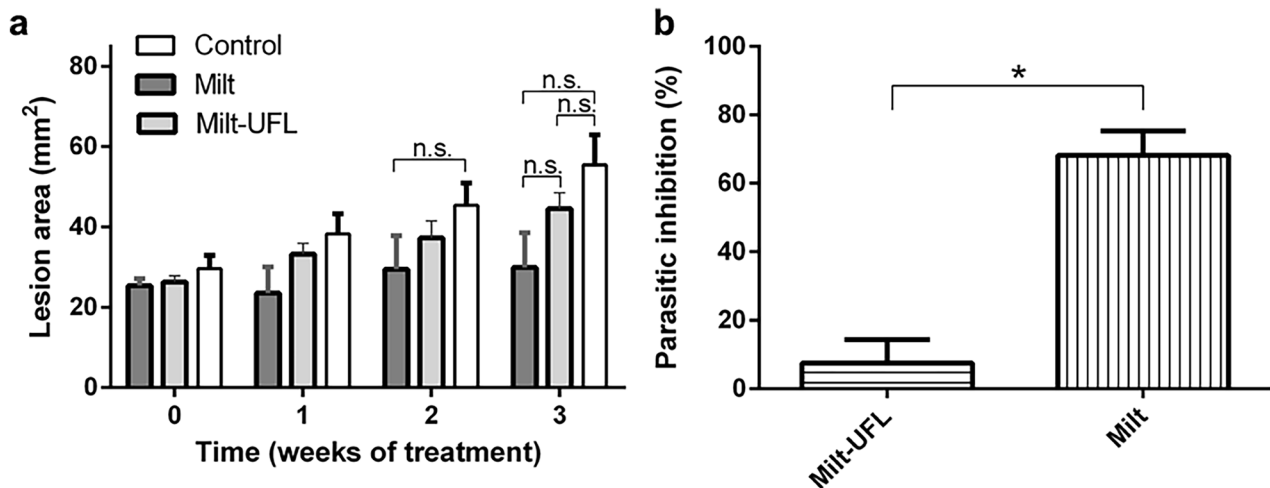

Fig. 3 In vivo efficacy of 0.015% Miltefosine (Milt) hydrogels. **a** Lesion area as a function of time for animals without any treatment (white bars), treated with gels containing only Milt (dark grey bars) and treated with gels containing ultra-fluid liposomes + Milt (lipid/drug molar ratio 7.33) (light grey bars). **b** Parasitic inhibition meas-

ured at the end of treatment in lesions of animals treated with gels containing only Milt or ultra-fluid liposomes + Milt. Treatments were dispensed once a day, 5 days a week during 3 weeks. $N = 5$. *$p < 0.05$
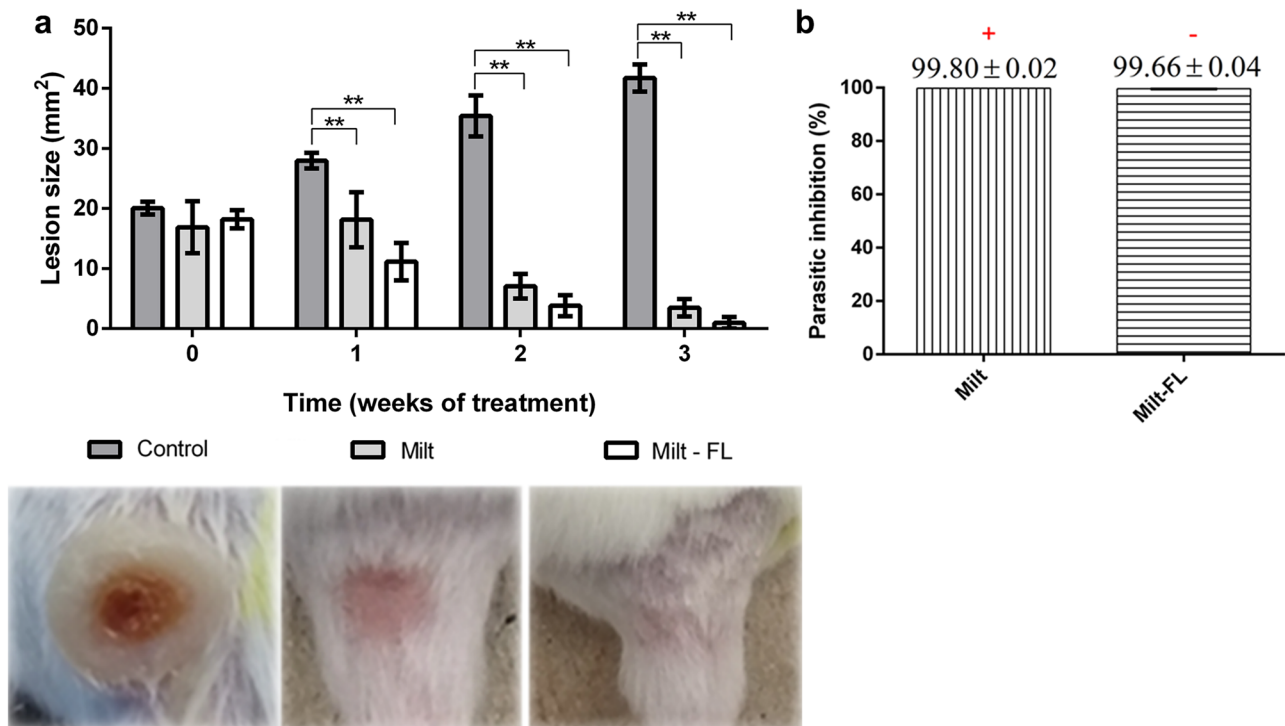
Fig. 4 In vivo efficacy of 0.5% Miltefosine (Milt) hydrogels. **a** Lesion area as a function of time for animals without any treatment (dark gray bars), treated with gels containing only Milt (light grey bars) and treated with gels containing fluid liposomes + Milt (lipid/drug molar ratio = 0.23) (white bars). **b** *Leishmania* amastigotes count reduction (parasitic inhibition) in lesion regions at the end of treatment. Symbols +/- indicates de viability of the remaining parasites.

Notice that, in animals treated with fluid liposomes + Milt, the re-culture in USMARU was negative, indicating no remaining viable parasites. Photographs of the lesions are representative of each group at the end of treatment. Treatments were administered twice a day, 5 days a week during 3 weeks. Statistical test: Mann–Whitney, $N = 5$. **$p < 0.004$

One month after end of treatment, all the animals recovered the hair (Fig. 5b) and had a normal-looking skin at the site of the lesion. It is also notable that mice treated with Milt-FL recovered the hair faster, since the length of hair at the lesion site was greater than in the mice treated with Milt. Figure 6 shows histological cuts of the lesion sites of an untreated control animal and of animals treated with Milt 0.5% with or without liposomes. Figure 6a shows how, in an untreated animal, the border of the ulcer is thickened and the tissue below the skin is filled with a granuloma formed by infected macrophages. The great amount of amastigotes present can be observed in Fig. 6b (black arrows). Lesions treated with Milt and Milt-FL showed complete re-epithelisation, without remaining infiltration. The epidermis looks normal and no evident fibrosis can be observed (Fig. 6c–f).

Immunoglobulins and hepatic enzymes at the end of treatment

Serum immunoglobulins were measured 1 month after the end of treatment with Milt at 0.5% to see the response of the immunological system to the infection. IgG1 is associated to a Th2 response and with the progression of the disease. On the other hand, IgG2a is associated to a Th1 response and is related to the cure and the elimination of the parasites [57–60]. The ratio IgG2a/IgG1 considers both responses: a higher value indicates a tendency to cure. Figure 6 g shows the results. One month after the end of treatment, the animals show IgG2a/IgG1 ratios that are not significantly different to control (untreated) animals, although animals treated with Milt plus liposomes have a slightly higher ratio than animals treated only with Milt.

To check for possible damage to the liver caused by the treatments, hepatic enzymes GOT and GPT were measured (Fig. 6h, i). The control group (without treatment) showed normal values for BALB/c mice: 100 U/l for GOT and 30 U/l for GPT [61, 62]. Serum from animals treated with Milt or Milt-FL contained similar enzyme values.

Physico-chemical characterisation of dispersions

We evaluated size, shape and stability upon storage of dispersions. Based on the results of the in vivo experiments, these measurements were performed in the dispersions that showed the highest efficacy: dispersions containing pure Milt at 0.5% and dispersions containing Milt-FL at PL/Milt molar ratio of 0.23; Milt 0.5%.
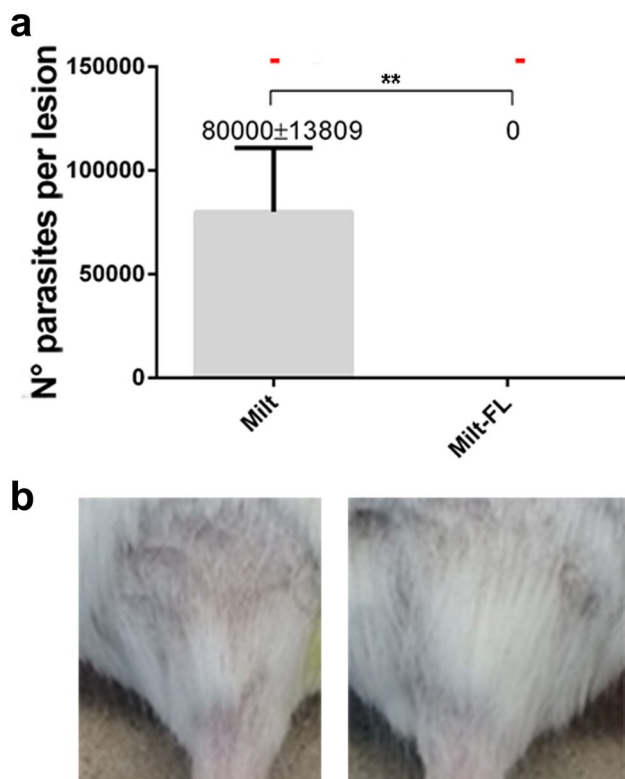
a

b

Fig. 5 In vivo efficacy of 0.5% Miltefosine (Milt) hydrogels 1 month after finishing treatment. **a** Number of parasites per lesion in animals treated with gels containing Milt or fluid liposomes + Milt. Symbols ± indicates de viability of the remaining parasites. Notice that the re-culture of the lesion material in USMARU did not produce *Leishmania* promastigotes, indicating that the parasites remaining in the skin after 1 month were not viable. **b** Representative photographs of the lesion site of mice 1 month after finishing treatment with Milt (left) or liposomes + Milt (right). Notice complete re-epithelisation, lack of scarring and re-growth of hair

Upon visual inspection, the hydrogels were homogeneous, with no observable phase separation, precipitation or change of colour after 90 days at 4 °C. The gels were easy to spread and had a pH of 6.

DLS was used to measure particle size. For clarity, we only show results of DLS from gel samples, since aqueous samples behaved in a very similar way. Both in buffer and in gel, pure Milt formed particles (micelles) of 4–8 nm diameter that were stable within this range for the assayed period (Fig. 7d, e, black squares). Mixtures of Milt and liposomes showed, immediately after preparation, the presence of 150 ± 50 nm particles (with this size, and according to EM images, these are most likely liposomes) (Fig. 7d, e, red circles). After 10-day incubation, however, two size populations appear, changing in relative size with time. At day 11, particles of 70 ± 30 and 19 ± 5 nm are detected. At day 19 of incubation, these two populations have become smaller, with 50 ± 30 and 6 ± 2 nm. From day 25 until day 45, the larger particles have diameters in the range 800–200 nm, while the smaller particles remain around $10–12 \pm 3$ nm.

Milt and phospholipids from the hydrogels, as analysed by TLC, showed no signs of chemical change (the chromatogram remained unchanged) after up to 9-month storage at 4 °C (results not shown).

In vitro kinetics of Milt release

Results of Milt release kinetics experiments are shown in Fig. 7f–h. The percentage of drug released from the pure Milt sample is linearly proportional to time and could be best fit by using a zero-order model, with $R^2 = 0,997$ (Fig. 7g, h). When liposomes were present, release kinetics was best fitted with the Higuchi model (Fig. 7g, h, $R^2 = 0.9674$), which indicates that the drug diffusion follows Fick's law [63]. The slope of the lines (the rate constants) obtained when fitting both data sets with the Higuchi model is smaller for Milt-FL (3.56) than for pure Milt (12.54) (Fig. 7h).

Discussion

We designed two different kinds of liposomes to increase trans-epidermal penetration of Milt. In vitro efficacy of Milt against different *Leishmania* species has been quite extensively studied and our results with pure Milt coincide with the literature [19, 21, 24]. In this work, the most interesting in vitro result was the evidence that phospholipid liposomes at low lipid/drug molar relation diminish the cytotoxicity and produce a notable increase in the efficacy of Milt against *Leishmania* amastigotes. Notably, empty liposomes showed anti-*Leishmania* activity by themselves at micromolar concentrations. Our in vitro results suggest that phospholipids can have an effect by themselves on the parasite's survival, displaying a toxic effect on amastigotes that does not produce toxicity on the macrophages. There is a precedent in the literature of 25% parasitic inhibition with phospholipids at milimolar concentrations [64]. It has also been shown in the literature that phospholipids modulate macrophages and immune system activity, increase macrophages phagocytic capacity and enhance incorporation of molecules joined to them into the macrophages where parasites are located [65–67].

On the other hand, we found that lipid/drug molar ratios are determinant, since liposomal formulations produced different results in vitro at 0.23 vs 7.33 lipid/drug molar ratios. At 7.33 PL/Milt molar ratio, the liposomes decreased or produced no alteration in Milt efficacy. The addition of NaChol to these formulations enhanced the performance of the drug at the lower Milt concentrations assayed, although the differences were not statistically significant. Regarding a possible irritation effect on skin, the in vitro assay against
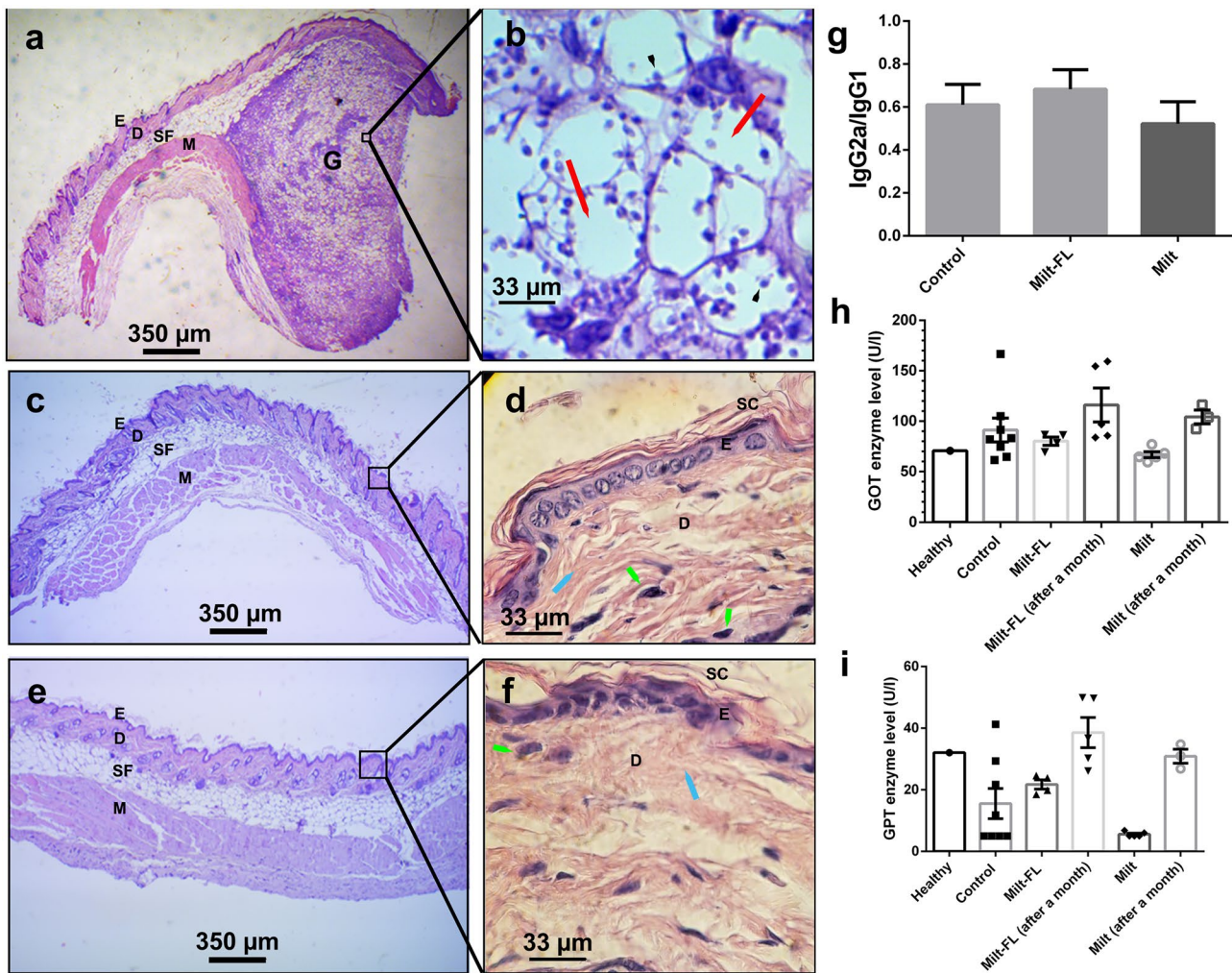
Fig. 6 Histology, hepatic enzymes and serum immunoglobulins after the end of treatment with Milt 0.5% gels. **a–f** Histology of mouse skin 1 month after the end of treatment. Shown are representative microphotographs of skin samples taken from the lesion site, stained with haematoxylin/eosin. **a** Lesion of a control animal (without treatment); **c** skin taken from the lesion site of an animal treated with Milt, **e** skin taken from the lesion site of an animal treated with fluid liposomes + Milt. **b** Magnification of **a**, where parasites (black arrows) can be seen inside parasitophorous vacuoles (red arrows).

d, **f** Magnifications of **c** and **e**, respectively, depicting the absence of parasites or signs of inflammatory infiltration. Green arrows point to nucleus of dermal cells, light blue arrows point to the extracellular matrix of the dermis. G granuloma, E epidermis, D dermis, SF subcutaneous fat, M muscle, SC stratum corneum. **g** Serum immunoglobulin levels. Measurements made 1 month after the end of treatment with formulations containing Miltefosine (Milt) and fluid liposomes + Milt (FL). **h** GOT. **i** GPT hepatic enzymes measured immediately at the end of treatment and 1 month later. $N = 5$

fibroblasts showed that none of the dispersions, including pure NaChol, produced cytotoxicity.

We studied the in vivo efficacy of Milt with and without liposomes and tried two Milt concentrations: 0.015 and 0.5% w/v. In order to facilitate topical application, we included the dispersions in a hydrogel. While the 0.015% hydrogels failed to produce a good result, we found that the formulations containing Milt at 0.5% were extremely efficacious.

Notably, the gel containing only 0.5% Milt (without any lipid or penetration enhancer) produced the apparent clinical cure of the animals after a 3-week treatment. While

this group of animals still had viable parasites in the skin immediately after the end of treatment, a month later, the animals were entirely cured, with no scar, regrowth of hair, and no (viable) parasites left at the lesion site. The histology showed an extraordinarily good skin healing, with no apparent fibrosis and no residual granuloma/inflammation.

The addition of fluid liposomes at low lipid/Milt molar ratio make the treatment with 0.5% Milt hydrogel equally efficient but faster. Already at day 8 of treatment, there is a difference in lesion size compared to animals treated with pure Milt, although the difference is not statistically significant. Furthermore, immediately after the end of
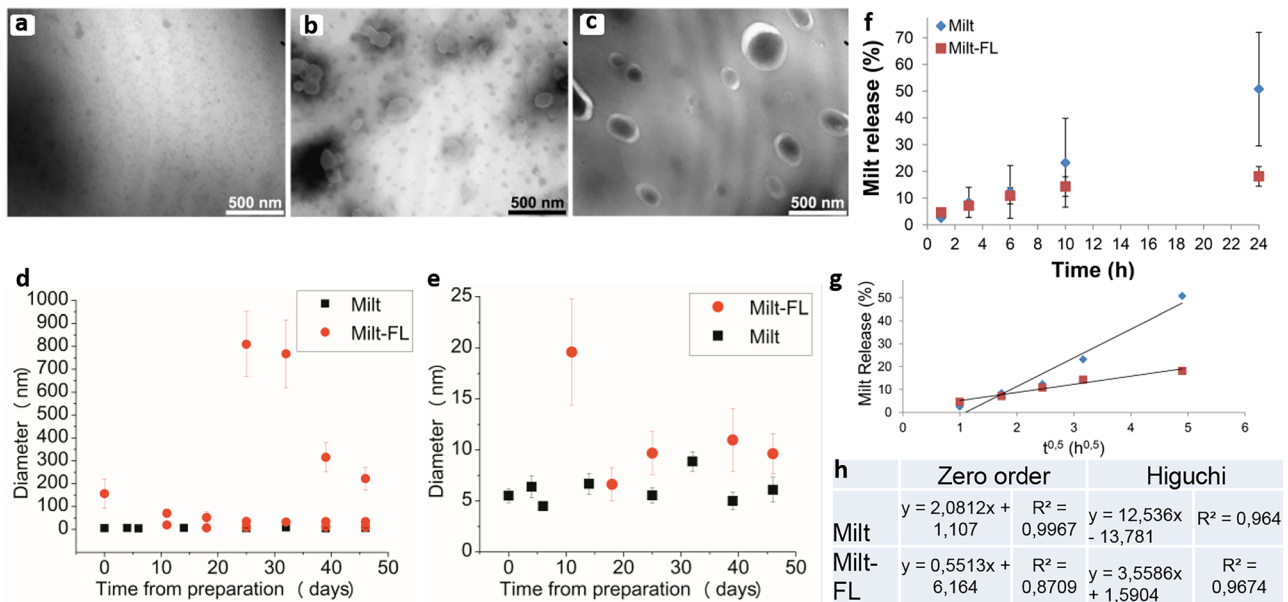
Fig. 7 Miltefosine dispersions characterisation. **a–c** Transmission electron microscopy images. Carbomer 940 hydrogel **a**; liposomes dispersion in buffer **b**; liposomes dispersion in Carbomer 940 hydrogel **c**. **d, e** Dynamic light scattering data of hydrogels (Milt 0.5%, lipid/drug molar ratio = 0.23) at increasing storage times. Hydrogels containing Milt or Milt + fluid liposomes (Milt-FL) dispersions, whole range of observed diameters **d**. Detail of data obtained at diameters between 0 and 25 nm **e**. **f–h** Kinetic data of Milt release in Franz cells through a policarbonate membrane (lipid/drug molar ratio = 0.23). Percentage of Milt released as a function of time from a pure Milt or a Milt + fluid liposomes dispersion **f**. Linear fit of drug release data following the Higuchi model **g**. Linear fitting results of drug release data using zero-order and Higuchi models. The slope of the line is the rate constant of release **h**. Results shown as mean $\pm$ EE ($n = 3$), $T = 35 \pm 1$ °C

treatment, no viable parasites can be recovered from the lesions, while this result can only be found with pure Milt a month after finishing the treatment. Animals treated with Milt 0.5% + liposomes also show macroscopical differences in healing speed. While both with and without liposomes the animals show complete re-epithelisation, immediately after the end of treatment those treated with liposomes show no scars at all and regrowth of hair, while animals treated with pure Milt still show small scars, that will take one more month to heal and become invisible due to hair regrowth.

The above observations coincide with our in vitro results showing an increased efficacy of lipid/Milt dispersions at low lipid/drug molar ratio (the same that was used in vivo). The mechanism of this effect may be due either to an effect of the fluid liposomes on the kinetics of Milt skin penetration or to a direct effect of the lipids themselves on the parasites [35, 68]. This last possibility however does not coincide with the fact that increasing the amount (molar ratio) of lipid in the in vitro experiments diminishes the efficacy against amastigotes (compare green and red striped bars in Fig. 2f).

Liposome fluidity is usually correlated to penetration enhancer/carrier performance through the skin [69]. This is why we included an edge activator (NaCholate) in one of our liposomes, making it ultra-flexible [70]. In the in vitro experiments, this detergent increased the efficacy against

amastigotes at some concentrations compared to pure Milt and did not produce cytotoxicity against fibroblasts. However, the in vivo experiments showed that the addition of NaChol was counterproductive. Ultra-fluid liposomes decreased the parasitic inhibition from 68 to 7.5% in our first experiment with Milt concentration of 0.015%. Based on this result and on parallel similar observations with a different drug, we decided not to test NaChol-containig liposomes any further.

Regarding systemic infection and toxicity, we did not find parasites in the spleen of treated animals. Also, hepatic enzymes were not elevated, which indicates there is no injury in the liver of the animals.

Milt has been shown to act both on the parasite and on the immune system of the host: in the parasite, it interferes in the synthesis of lipids [71, 72], impairs the metabolism of arginine [73] and produces cell death by oxidative stress [74]. In the host, it has been shown to have an immunomodulatory effect involving Th1 response, especially in animal models of visceral and diffuse cutaneous leishmaniasis [75]. In our experiments, Milt-FL did not produce a significant Th1 response, although IgG2a/IgG1 levels tend to be higher than in the control cases. Animals treated only with Milt show IgG2a/IgG1 levels lower than the control animals, although again the difference is not statistically significant. This

lack of clear effect of Milt may be due to our model. The Th1/Th2 balance is more complex in localised CL models than in visceral models, with high levels of Th2 cytokines present in healing localised lesions [75]. A future in vitro study of Th1 cytokine production like IFN-ϒ and IL-12 by infected macrophages should help to better understand the immunomodulatory response of Milt-FL.

In order to better understand the possible mechanism of action of the most efficacious formulations (containing 0.5% Milt and liposomes at lipid/Milt molar ratio = 0.23, without NaChol), we performed a physico-chemical characterisation of aqueous lipid dispersions and gels identical to those used in vivo. Regarding storage stability, upon visual inspection the hydrogels were stable after 90 days at 4 °C. Thin-layer chromatography also detected no change upon 90-day storage. Size distribution analysis however did show changes in phospholipid-containing samples. While pure Milt suspensions remained stable, with 4–8 nm micelles, in lipid/Milt mixtures the liposomes that had 150 ± 50 nm immediately upon preparation suffered changes in size distribution with storage time. After 10 days, already two size populations were present. These two populations varied in size with time, one with sizes compatible with liposomes (800–200 nm), the other with very small sizes most likely representing mixed Milt/PL micelles.

In vitro release experiments showed that Milt release through a polycarbonate membrane was quite fast, but it was slowed down when liposomes were added to the formulation. A decrease in release rate is found when comparing the rate of diffusion using the same theoretical model for both systems.

Milt is water soluble but amphiphilic, and so it will partition into the hydrophobic phase offered by liposomes. In the absence of an oily phase to partition, Milt forms micelles. In the presence of membranes, part of the Milt molecules will be dissolved in the water and part will be dissolved in the membranes, the exact amount depending on the membrane/water partition coefficient of Milt. This value ($K = C_m/C_w$) is approx. 10^5 for Milt [76], and so, in the sample containing liposomes, most of Milt molecules will be partitioned into the liposomes membranes [77].

In the pure Milt sample, only monomers and small (4–8 nm) Milt micelles are present at any storage time evaluated. Micelles could permeate the 100-nm pore membrane easily, and indeed the kinetics is best fitted with the simplest model, a zero-order model (the drug is released at a constant rate with time). In the presence of phospholipids on the other hand, molecules organise forming a mixture of monomeric Milt and lipid molecules, mixed Milt/PL liposomes of different diameters (150–800 nm) and mixed Milt/PL micelles, and the data is best fitted with a more complex model (the release of drug is linearly proportional to the square root of time). Since 150–800 nm liposomes

cannot spontaneously traverse the polycarbonate membrane, this would explain why the rate of release of Milt is smaller in the presence of liposomes. This result also suggests that the dissociation of Milt from lipids is the step that slows down and controls the delivery rate. This could influence Milt activity during the topical treatments by slowing down and extending in time the diffusion of Milt into the skin and could explain why the phospholipid-containing formulation is more efficacious that the pure Milt formulation.

Van Bocxlaer et al. reported that topical formulations of Milt at 6% induced skin irritation [33]. This fact could be related with the concentration used. In that sense, it is important to mention that in our experiments, we did not observe signs of irritation in the animals, neither in Milt nor in Milt-FL groups. Also, an in vitro experiment against fibroblasts did not show cytotoxicity of any of the formulations that we used, at any concentration tested, even in those containing NaChol.

Simultaneously with our work and corroborating our results, two very recent reports have been published on the activity of topical Milt formulations on CL induced by other species of *Leishmania* [78, 79]. Neira et al. describe the efficacy of a Carbopol gel containing Milt in BALB/c mice infected with *L. (V.) braziliensis* and *L. (V.) panamensis*. These authors used 10% DMSO as a penetration enhancer. This is a rather high amount of DMSO, raising concern for irritation/toxicity. Kavian et al. report the effect of topical formulations containing Milt on BALB/c mice subcutaneously infected with *L. (L.) major* [78]. These formulations contained PC and cholesterol liposomes (our liposomes contained no cholesterol) and were dissolved in buffer (a low-viscosity medium). The formulation containing 0.5% Milt failed to completely inhibit parasite burden, and only the formulations containing 2 and 4% Milt produced good parasite inhibition. No mention is made to the possible irritation produced by the higher Milt concentrations. The presence of cholesterol induces an increase in microviscosity and bending rigidity in phospholipid membranes, inducing a liquid-ordered state in the membrane [80]. It is possible that the presence of cholesterol acts against the trans-epidermal penetration of Milt. This would explain the need for higher concentrations of Milt to obtain a good therapeutic effect.

As discussed above, in the gel-containing liposomes, most of Milt molecules will be partitioned into the liposomes membranes. There is a certain agreement in the literature that, when in contact with skin, liposomes can only penetrate intact the first few microns of the stratum corneum (SC), thereafter breaking down, and the molecular components diffusing into the skin by themselves [34, 81–84]. In our view, it is not the presence of liposomes as intact carriers to the dermis what aides the efficacy of Milt, but rather the effect of the particular phospholipid molecules used to produce the liposomes: unsaturated phospholipids make skin

lipids more fluid, therefore diminishing the barrier function of the skin and acting as a penetration enhancer [85].

However, a more nuanced effect may be at play here. As shown before [34], liposomes can both increase a drug´s penetration into the skin and make the penetration slower. We speculate that in the present case, liposomes accumulate on the first few microns of the SC, acting as a depot of material slowly releasing Milt and phospholipids. The lipids however would also aid the penetration of free Milt molecules by fluidising the SC membranes. At the same time, as suggested by our in vitro results, phospholipids could have an effect by themselves on the parasite's survival.

Concluding remarks

The present work demonstrates that a topical treatment with pure Milt at a concentration of 0.5% w/v is highly effective against cutaneous leishmaniasis induced by *L.* (*L.*) *amazonensis* in vivo*,* and that the addition of fluid liposomes enhances the anti-parasitic effect of Milt, accelerating the clinical cure and the complete elimination of parasites. The combination of the use of a gel to enhance viscosity and of fluid liposomes as penetration enhancers/release modulators seems promising, since it avoids the risk of losing the formulation applied on the skin due to low viscosity and also avoids the need to use a harsh penetration enhancer such as DMSO.

Another important issue is whether this formulation will work in animals with a skin thicker than that of mice, such as humans. Human skin is approximately 4 times thicker than mouse skin. It is therefore of the outmost importance to try the formulation in other mammals with thicker skin in order to evaluate the need of an adjustment of Milt or liposomes concentration, the length of treatment, etc.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication All authors have consented to the publication.

Animal studies All institutional and national guidelines for the care and use of laboratory animals were followed.

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