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Case Study

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A Case of Cutaneous Leishmaniasis Cured by a Combined Treatment of Topical Miltefosine and Injectable Amphotericin B

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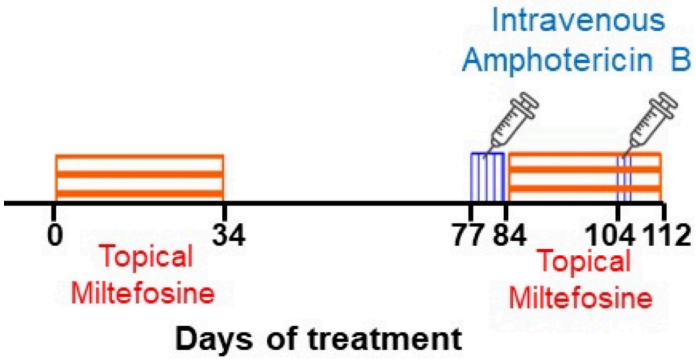
Graphical abstract



Cutaneous leishmaniasis cured by the combination of topical Miltefosine and standard Amphotericin B.

Abstract

A male rural worker with cutaneous leishmaniasis (CL) was initially treated with repeated cycles of Meglumine Antimoniate (Glucantime®) and two cycles of Amphotericin B deoxycholate during a three-year period. However, the patient remained with two active lesions. An experimental topical treatment with liposomal Miltefosine was tested. The efficacy was determined from the MD's inspection of the patient and analysis of the lesions' photographic images, lesions samples culture, and anti-Leishmania antibodies quantification. The parasites from the patient were isolated. The parasites were typified as Leishmania braziliensis, and their susceptibility to Miltefosine, Meglumine Antimoniate, and Amphotericin B was measured. The wound healing was initially good with the topical treatment, but the clinical cure was achieved only with a combination of the topical liposomal Miltefosine and intravenous Amphotericin B. Of note, the treatment efficacy was strongly correlated with the degree of stress suffered by the patient. To our knowledge, this is the first report on using a topical Miltefosine formulation on a CL patient.

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Keywords: American cutaneous leishmaniasis, topical treatment, Miltefosine, liposomes, nanoparticles, gel, clinical case

Rationale, Purpose, and Limitations

Cutaneous leishmaniasis is an orphan parasitic disease that causes important suffering, especially among rural workers in poor/underdeveloped countries. Standard treatments are inadequate as they are systemic, toxic, and painful and require trained medical personnel as well as type 2/3 hospital infrastructure to apply treatment and manage side effects. The development of resistance is an increasing concern. An efficacious topical treatment would be a great gain for patients and for the healthcare system of affected countries: it could be self-administered, would not produce pain or systemic secondary effects, would increase patient compliance, and would be overall a smaller financial burden to the healthcare system. We have developed a topical treatment containing liposomal Miltefosine (MIL) that showed excellent efficacy in preclinical trials in mice. Our objective was to evaluate the efficacy of a MIL gel obtained as a compounded formulation in a patient with resistant CL. A limitation of the study is that we did not have access to the patient from the first diagnosis.

Introduction

Leishmaniasis is a parasitic disease transmitted by the bite of sandflies infected with *Leishmania (L.)* parasites. More than 20 species of *Leishmaniasis* give rise to three kinds of disease: visceral, cutaneous, and mucocutaneous. Cutaneous leishmaniasis (CL) is the most frequently occurring form, with an estimated annual incidence of 1,000,000 new cases, in contrast to the 30,000 new cases of visceral leishmaniasis reported worldwide [1]. Mucocutaneous leishmaniasis (MCL) is a disabling variant due to its destructive impact on the mucous membranes of the nose, mouth, and throat cavities. Visceral leishmaniasis affects internal organs and can be fatal if untreated.

CL produces skin lesions usually appearing on exposed body parts, which may either spontaneously heal or progress into ulcers. Often, the treatment of CL results in unsightly scars. In cases where the parasite remains within the body, it can lead to disease relapse or even transition into MCL. CL can also be categorized as American or New World CL (NWCL), affecting the Western Hemisphere (mainly caused by *Leishmania braziliensis, amazonensis, mexicana,* and *guyanensis*), and Old World CL (OWCL), which occurs in the Eastern Hemisphere (mainly caused by *Leishmania major, aethiopica* and *tropica*) [2]. The conventional treatment for CL involves pentavalent antimonials, such as N-methyl Glucamine Antimoniate (Glucantime®), administered intravenously or intramuscularly at a dosage of 10-20 mg/kg/day continuously for 20 – 30 days [3]. This treatment is generally well tolerated, although it may produce pain at the injection site, gastrointestinal disturbances, arrhythmias, pancreatitis, and joint stiffness. In cases of treatment failure, Amphotericin B (AmB) is employed as a second option; administered intravenously at a daily dose of 1-4 mg/kg/day, it is contraindicated in patients with heart problems, liver disease, and nephropathy. A less toxic liposomal AmB formulation (Ambisome®) is commercially available; however, its use is limited in underdeveloped countries due to its high cost. Pentamidine Isethionate is reserved for cases where antimonials prove ineffective and AmB results are excessively toxic. Miltefosine is the first oral drug approved for the treatment of CL, although it has shown to be teratogenic in preclinical trials, limiting its use to males, children above 12 years old, and women using contraception [3–5].

In the Americas, local treatments for CL are constrained to specific regions. The World Health Organization and the Pan American Health Organization have advocated their usage in a recent Technical Report, primarily due to the recognition that no treatment can completely eradicate infections. Local treatments are preferred owing to their reduced toxicity and greater patient acceptance [6]. Topical formulations containing 15% Paromomycin or 15% Paromomycin + 0.5% Gentamicin have exhibited a cure rate of 75%-94% in uncomplicated patients with *Leishmania major* and *Leishmania braziliensis* [7–9]. A cream incorporating the immunomodulator Imiquimod at a concentration of 5% enhanced the

efficacy of systemic Meglumine Antimoniate in patients with NWCL, but not in patients with OWCL. Topical formulations of Amphotericin B, including lipid-based ones, failed to yield significant efficacy results [10].

A 6% MIL gel demonstrated poor efficacy in preclinical studies and provoked considerable irritation in BALB/c rodents with *Leishmania major* [11]. However, the same concentration reduced the parasite burden by 85-99% in another assay with mice infected with *Leishmania mexicana* and *Leishmania major* [12].

Subsequent investigations employed a 0.5% MIL gel, achieving efficacies of 85-99% in BALB/c animals infected with *Leishmania braziliensis* and *Leishmania panamensis* [13]. Preliminary studies also showed that liposomal MIL formulations containing the drug at 4% enhance the therapeutic outcome compared to conventional approaches in mice [10]. A gel containing ultra-deformable liposomes with MIL and apigenin at 0.1% proved efficacious against *Leishmania mexicana* in BALB/c mice [14].

Previously, we developed a liposomal MIL gel that was topically tested in BALB/c mice infected with *Leishmania amazonensis*. This experimental formulation exhibited no evidence of local irritation or hepatic toxicity. Remarkably, the liposomal formulation resulted in a 99.9% reduction of parasite burden, achieving sterilization of the lesion area, complete re-epithelialization with no signs of scarring, even at the histological level, and no relapse after 30 days [15].

In this work, we report the case of a male rural worker with cutaneous leishmaniasis with two active resistant lesions that were treated with an optimized topical MIL formulation. The patient was treated with our formulation and was closely followed. We identified the parasite species, measured anti-parasite antibodies, and the susceptibility of the isolated parasite to the drugs used on the patient. To the best of our knowledge, this is the first report on using a topical formulation of MIL on a CL case.

Experimental design

In the realm of parasitic disease management, such as leishmaniasis, treatment ineffectiveness commonly poses a significant challenge. From the parasite's standpoint, aside from concerns regarding safety and treatment efficacy, the emergence of drug resistance is often regarded as a key factor contributing to treatment failures. Nevertheless, the reliability of *in vitro* drug susceptibility tests for assessing this phenomenon remains uncertain. Some studies suggest a correlation between treatment outcomes and the parasite's susceptibility to drugs, while others fail to establish such a connection. Consequently, the evaluation of clinical results post-treatment assumes paramount importance.

The objective of this work was to attempt to cure the patient-resistant lesions using a topical MIL formulation. Of note, this drug had not been previously administered to the patient. Given the high efficacy of our topical formulation in mice, we hypothesized that the MIL gel would cure the patient while avoiding toxic systemic side effects and the painful injections of the standard treatments. In light of these considerations, the patient started treatment with a formulation containing liposomal MIL gel that was prepared based on our previous preclinical experience. To avoid interference in interpreting the results, the treatment was scheduled 38 days after the last pre-treatment dose with Amphotericin B (AmB).

Materials and Methods

Liposomal Miltefosine gel preparation

The hydrogel formulation was prepared using liposomes of unsaturated phospholipids at 0.25% w/v (Avanti Polar Lipids, Alabama, USA), Miltefosine 0.5% w/v or 1% w/v (MAPCHO® from Avanti Polar Lipids, Alabama, USA) and Carbomer 934-P NF (Cb) as the gelling agent at 1% w/v (Saporitti®, Bs. As. Argentina). Milli Q water was used as the vehicle, and the pH was adjusted with NaOH 1M to 7.40 ± 0.05.

A Cb aqueous dispersion was prepared by magnetic stirring. The MIL aqueous solution, as well as the dispersion of the liposomes, were added to the gel, and the pH was adjusted with NaOH 1 M and an adequate pH meter to measure viscous samples. The multilamellar liposomes were prepared by thin film hydration: phospholipids were dissolved in chloroform : methanol (2:1) and put in a glass flask; the solvent was evaporated by

continuous rotation under a nitrogen atmosphere, followed by 3 hours of vacuum. The thin film formed by the lipid was hydrated with sterile MilliQ water (3 mL, 75 mg/mL final concentration).

After preparation, the formulation was sterilized by autoclave (20 min, 121 ℃). The hydrogel was then aliquoted under a laminar flow hood in sterile 2 mL syringes and stored at 4 ℃. The compound formulation was produced in the Pharmacy of Hospital Nacional de Clínicas of Córdoba following Good Laboratory Practices as regulated by the Argentinian National Ministry of Health [16].

Treatment and clinical evaluation

1. Pre-treatment phase

We present the case of a 52-year-old male rural worker from Orán, Salta, Argentina, who was first diagnosed with CL in June 2020. At that time, he exhibited multiple ulcers in the left knee and thigh. Subsequently, he received two rounds of treatment (10 mg/kg/day for 21 days each time) with Meglumine Antimoniate (Glucantime®). Additionally, he received one course of treatment with Amphotericin B deoxycholate (Amphotericin from Richet) at a dosage of 0.6 mg/kg/day, which had to be discontinued on the 6th day due to cardiac toxicity. During the month of January 2021, treatment was provided for a secondary bacterial infection affecting the lesions. In June 2022, several lesions still remained active, and swabs collected from these lesions revealed amplification of the kDNA of *Leishmania* spp via kPCR. Furthermore, anti-*Leishmania* IgG was detected in the blood serum. Consequently, the patient underwent a third round of treatment with Glucantime® (10 mg/kg/day for 21 days), followed by AmB deoxycholate (0.6 mg/kg/day). Once again, the AmB treatment had to be halted after 6 days, this time due to hypokalemia and cardiac toxicity. At the end of this phase, two lesions remained active (see Supporting Figure 1), and swab analysis continued to show amplification of kDNA of *Leishmania* spp, and anti-*Leishmania* IgG was still reactive in the blood serum. In addition, when aspirated material from the lesion edge was inoculated into a Difco blood agar (USMARU) medium containing 20% of defibrinated rabbit blood, promastigotes developed after five days of culture, indicating the presence of viable amastigotes in the lesion. The patient did not present chronic diseases, did not consume other medications, or had a history of allergic reactions. His quality of life, however, was seriously affected by the long history of CL treatment failure.

2. Ethical considerations

This is a case of an off-label drug use that has been prepared as a compound. According to the WHO, Argentinian off-label use regulations, and to other Argentinian and European jurisprudence (see [17]), in the absence of high-quality evidence (complete clinical trials), a medical doctor can decide to use a drug off-label when the patient's condition is serious, and there is evidence supporting the potential benefit (especially if the expected benefits exceed the potential risk); standard therapy is considered inadequate to achieve the desired result; patients have been adequately informed about the possible benefits and risks of off-label use and have given their consent; active and appropriate monitoring of medication safety is implemented, including mechanisms to rapidly identify and manage adverse events. Similar considerations are expressed in other international literature; see, for example, Gazarian et al. in the case of "exceptional use, justified by individual clinical circumstances" [18].

All of the above considerations were positive in our case. Thus, it was within the powers of the MD to decide on the use of the off-label treatment. The intervention of an ethics committee would not have been appropriate since this was a particular practice for only one particular patient and was not to be extended to others. It is important to highlight that we had very positive preclinical data (where no adverse side effects had been observed, and the efficacy had been 99.9%); the medication was used as a "last resort therapy" since the patient was resistant to all available therapies; and no other licensed treatment was available. Also, the risks-benefits balance of the treatment was evaluated, taking into account that the formulation was intended for topical application (thus minimizing its potential systemic side effects).

One week before initiating treatment, the patient was provided with an informed consent document to take home for review. The patient is fully literate. In this instance, the patient received a comprehensive explanation from his physician regarding the pertinent details of the experimental treatment, including its outcomes in animal studies and potential side effects. On the first day of the treatment protocol, the patient signed the informed consent form in the doctor's presence.

3. Efficacy evaluation of treatment

3a. Lesion area quantification and visual evaluation

To initiate the treatment we decided to employ the same concentration of Miltefosine (MIL) as utilized in our preclinical studies (0.5% w/v). The application of the gel was scheduled twice daily for a period of 30 days. The volume to be administered to each lesion was determined by establishing a direct proportionality with the largest diameter of the lesion. This approach ensured an appropriate dosage tailored to the size of the individual lesions. This calculation was performed as follows:

$$Vol\_patient = Vol\_mouse \times (d\_patient / d\_mouse)$$

Where:

Vol_patient represents the volume to be applied to the patient's lesion.

Vol_mouse corresponds to the volume applied to the lesions in mice.

d_patient denotes the largest diameter of the patient's lesion.

d_mice indicates the largest diameter of the lesions in mice.

Lesion area was calculated using the area of an ellipse, and was monitored weekly through photography and recording by the MD:

$$Lesion\ area = (L \times W \times \pi)$$

Where:

L represents the larger radius of the lesion.

W represents the radius of the lesion taken at 90° to L.

The lesions were categorized based on their severity from the analysis of lesion appearance, as detailed in Table 1. The MD determined the scores through visual inspection of the lesions and examination of photographs. The progression of lesion scores over time was plotted for analysis.

3b. Determination of anti-Leishmania antibodies

To detect anti-*Leishmania* IgG in the patient's serum samples, an Enzyme-Linked Immunosorbent Assay (ELISA) was employed. The method consists, briefly, of the following steps:

Sensitization of Plates: Plates were sensitized by incubating them overnight at 4 ℃ with 100 µL of soluble crude antigen enriched with membrane crude antigen extracted from promastigotes of *L. (V.) braziliensis* (MHOM/AR/02/OLO1) at a concentration of 1.8 µg/mL. This antigen was diluted in a bicarbonate/carbonate coating buffer, pH 9.6.

Washing: Unbound antigen was removed by washing the wells three times with a washing buffer, which contained 0.05% Tween 20 in 0.01 M phosphate-buffered saline, pH 7.0 (PBST).

Blocking: To prevent non-specific binding reactions, 300 µL of a 5% PBS-skim milk solution was added to each well.

Sample Dilution: Serum samples were diluted at a ratio of 1/70 in PBST containing 1% skim milk. Then, 100 µL of this diluted serum solution was added to each well.

Incubation: The plates were incubated at 37 ℃ for one hour.

Washing: After incubation, the plates were washed four times with PBST.

Secondary Antibody Addition: Subsequently, 100 µL of goat-antihuman polyvalent Immunoglobuline (G)-Peroxidase conjugate (Invitrogen) diluted at a ratio 1/4500 was added to each well.

Following the final incubation at 37 ℃ for 30 minutes and four subsequent washes, the plate was subjected to the revelation step using a solution consisting of 0.1 mg/mL of 3,3′,5,5′-tetramethylbenzidine (TMB), 0.1X dimethyl sulfoxide (DMSO), citrate phosphate buffer (pH 5), and 0.03 volumes of H_2O_2 (hydrogen peroxide 0.03%). The enzymatic reaction was allowed to proceed until appropriate color development.

Table 1. Score values assigned to each lesion stage.

Severity score	Designation	Description
6	Necrotic ulcer (Ulcer + necrosis)	Ulcer pit, dry and regularly rounded high edges, lesions displaying a dark red/black color in the center.
5	Flattened edge + inflammation	Flat wound, edges are flattened around the entire perimeter of the lesion. Signs of inflammation are evident.
4	Flattened edge (80% erythema)	Flat wound, edges are flattened around the entire perimeter of the lesion. Erythema present in 80% of the lesion area.
3	Flattened edge (20% erythema)	Flat wound, edges are flattened around the entire perimeter of the lesion. Erythema present in 20% of the area of the lesion.
2	Granulation tissue 80% (Cure tendency)	New connective tissue covering 80% of the total lesion area. This tissue appears red to pink in color, is moist, and exhibits a bumpy (granular) texture.
1	Granulation tissue 20% (Cure tendency)	New connective tissue covers 20% of the total lesion area. This tissue is red to pink in color, moist, and bumpy (granular) in texture. The rest of the lesion is covered by new epithelium.
0	Cicatrization	Complete healing. The whole lesion is covered by new epithelium.

To halt the reaction, 0.5 N H_2SO_4 (sulfuric acid) was employed.

The absorbance values (ODs) resulting from the reaction were measured using a spectrophotometer at 450 nm wavelength. This allowed for the quantification of anti-*Leishmania* IgG levels in the serum samples, providing data for evaluating the patient's immune response [19].

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3c. PCR amplification, sequencing, and species identification

Parasites were isolated by using USMARU-PBSS medium, following the procedure previously described by Marco *et al.* [20]. DNA extraction was performed using the Higuchi method, and then a specific hsp70 gene fragment was amplified using the PCR-N (593 bp) protocols outlined by Montalvo *et al.* [21]. The primers were: F25: GGACGCCGGCACGATTKCT and R617: CGAAGAAGTCCGATACGAGGG.

The reaction mix consisted of 4 µL of the template DNA in a total volume of 25 µL with final concentrations of 1X T-Holmes Buffer, 1 U of T-Holmes DNA polymerase, 200 µM of each deoxyribonucleoside triphosphate (dNTP), 2.5 mM of MgCl2, 0.8 µM of each primer, and 0.1 mg/mL

of reaction enhancer (INBIO Highway). The amplification conditions were: denaturation at 95 ℃ for 5 min; followed by 35 cycles of denaturation at 94 ℃ for 40 s, annealing at 61 ℃ for 1 min, extension at 72 ℃ for 1 min; and a final extension step of 10 min at 72 ℃. Negative controls were consistently included in the experiments, along with a positive control, comprising 100 fg of DNA from the *L. braziliensis* strain MHOM/BR/75/M2903.

Thermal cycling was performed in an ESCO Swift™, Maxi Thermal Cycler Block (ESCO Technologies Inc., Lab Division, MO, USA). The PCR products were visualized through 2% agarose gel electrophoresis stained with GelRed (Biotium, EE.UU.) for 40 minutes at 80 V. The resulting product was sent for sequencing, following the instructions from the Capillary Electrophoresis Sequencing (CES) service provided by Macrogen Inc. (https://dna.macrogen.com/). The obtained sequences were compared with those containing available information from the hsp70 gene region used as a molecular marker (1116 bp) on GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and in the database constructed by Van der Auwera and colleagues [22]. Alignments were constructed using the MEGA 11 software [23]. Finally, the Neighbor-Joining (NJ) distance method with 1000 bootstrap replicates was applied using the MLSTest software Field [24], considering the average distance between coexisting bases at heterozygous sites in order to determine the species.

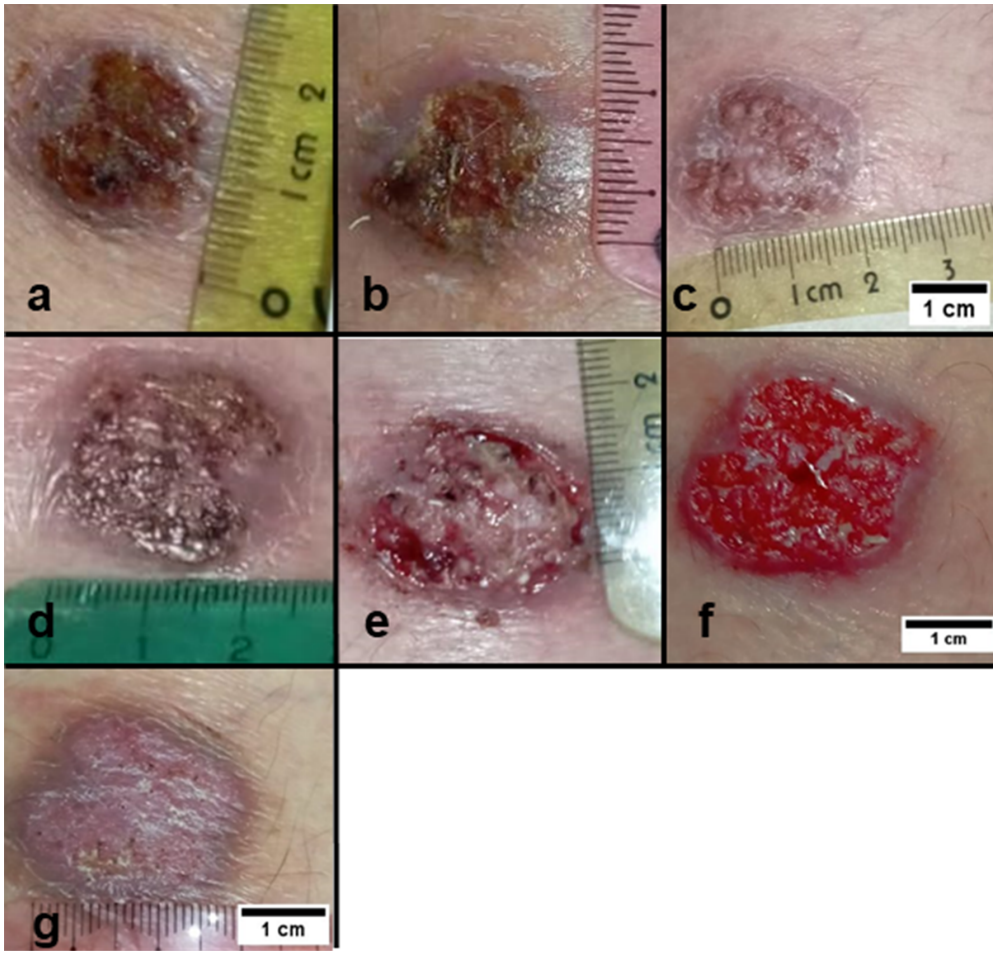

Figure 1. Photographs of Lesion 1. a) Day 1, before treatment; b) day 8 (beginning of re-epithelialization from the borders of the lesion and diminished erythema); c) day 21 (re-epithelialization is observed both from the borders and inside the lesion, no erythema); d) day 35 (scab), e) day 43 (the milky and yellow liquid indicates over-infection), f) day 77 (the scab came off, area increase), and g) day 100 of treatment (complete re-epithelialization).

3d. In vitro parasite susceptibility to Glucantime, Amphotericin B, and Miltefosine

Raw 264.7 macrophages (200,000/mL) were seeded into Lab-Tek chamber slides and allowed to adhere for 3 h at 37 ℃ in a 5% CO2 95% air mixture.

Parasites isolated from the patient's lesions were cultured in RPMI 1640 medium and harvested at stationary phase to infect cells at a ratio of ten parasites per macrophage. The infected cells were subsequently incubated at 34 ℃ in a 5% CO2 95% air mixture for 24 h.

Following the infection period, the cells were washed with pre-warmed PBS to remove free parasites. A fresh medium with varying concentrations of Glucantime (ranging from 40 to 480 µg/mL), AmB (ranging from 0.01 to 0.35 µg/mL), and MIL (ranging from 0.12 to 4 µM) was added to each well. The positive controls were infected cells cultured in a complete RPMI-1640 medium only. The range of concentrations used for each drug was selected to remain below the CC50 (50% cytotoxic concentration) of the respective drug in macrophages.

Results and Discussion

Clinical cure was achieved by a combination of MIL gel and intravenous AmB

On day 1, the patient presented 2 lesions. The largest, Lesion 1, was in the thigh and measured 2.5 x 2.0 cm (Figure 1a); the smallest, Lesion 2, was in the knee.

Lesion 1

Except for the first application, the formulation was self-applied by the patient throughout the treatment. Lesion 1 was treated with 1 mL of topical 0.5% liposomal MIL twice daily for the first 21 days. On the first application, the MD observed that this volume was excessive for the area to be treated, so it was decided to divide the application in two, applying 0.5 mL, waiting 40 minutes for the gel to dry, and then applying the rest of the dose. The patient described a mild burning sensation (rated as 2 on a scale of 1 to 10) during the first 2-3 seconds after the application of the gel on the lesion.

On day 21, the lesion showed significant improvement (Figure 1c), with the smallest area recorded since starting the treatment (Figure 2a).

As shown in Figure 3, the improvement of the lesion in this period is also evident in the score assignment.

From day 22 to day 31, the patient applied half the indicated dose (0.5 mL), and the lesion increased its area (Figure 2a). From day 31 to 34, the original treatment was resumed, and the patient applied 1 mL of the gel twice daily.

On day 35, the lesion area was 44% smaller than on day 1 (Figure 2a), and a tendency to cure was evident (Figure 3): the lesion shows a scab but no suppuration or blood (Figure 1d). Unfortunately, at this time, the formulation administration had to be interrupted as there was a lack of access to MIL in Argentina.

On day 43, the lesion showed signs of over-infection (Figure 1e). For this reason, the patient received oral treatment with Sulfamethoxazole (800 mg) + Trimethoprim (160 mg) (Bactrim Forte®) twice a day for 5 days. On day 57, the lesion was still infected with bacteria, and Amoxicillin (875 mg) and Clavulanic acid (125 mg) were administered once a day for 15 days.

On day 5 of this second antibiotic treatment, the lesion area decreased by 40% to 2.8 cm2 (Figure 2a); however, on day 72 (still under antibiotic treatment), the lesion area grew again to 4.6. After finishing antibiotic treatment on day 77, the lesion area was the biggest since the beginning of treatment (9.2 cm^2, 2.3 times bigger than on day 1), and the scab came off (Figure 1f and 2a). For this reason, the MD decided to apply intravenous AmB (0.6 mg/kg/day). The patient received 6 days of this treatment, which produced joint pain, tachycardia, high creatinine levels (2.3 mg/dL), high urea levels (0.76 g/L), hypokalemia (3.08 mEq/L) and hypochloremia (92 mEq/L). In summary, MIL gel at 0.5% improved the lesion considerably, but an over-infection with bacteria and the environmental/secondary conditions conspired against the cure. It was thus decided to apply two short treatments of intravenous AmB simultaneously with MIL gel at 1%. This combination finally produced the re-epithelialization of the ulcer.

The treatment with AmB was suspended, and a second treatment with the topical MIL formulation was started on day 84. To decrease the volume of gel to be applied, we decided to double the MIL concentration to 1% w/v. The patient applied 0.6 mL of this 1% MIL gel twice daily for 28 days (day 84 - day 112). Simultaneously, from day 104 to 106, 3 more doses of AmB were administered, which again produced renal toxicity (creatinine of 1.64 mg/dL). At day 100, lesion 1 was completely re-epithelialized (Figure 1g and Figure 3).

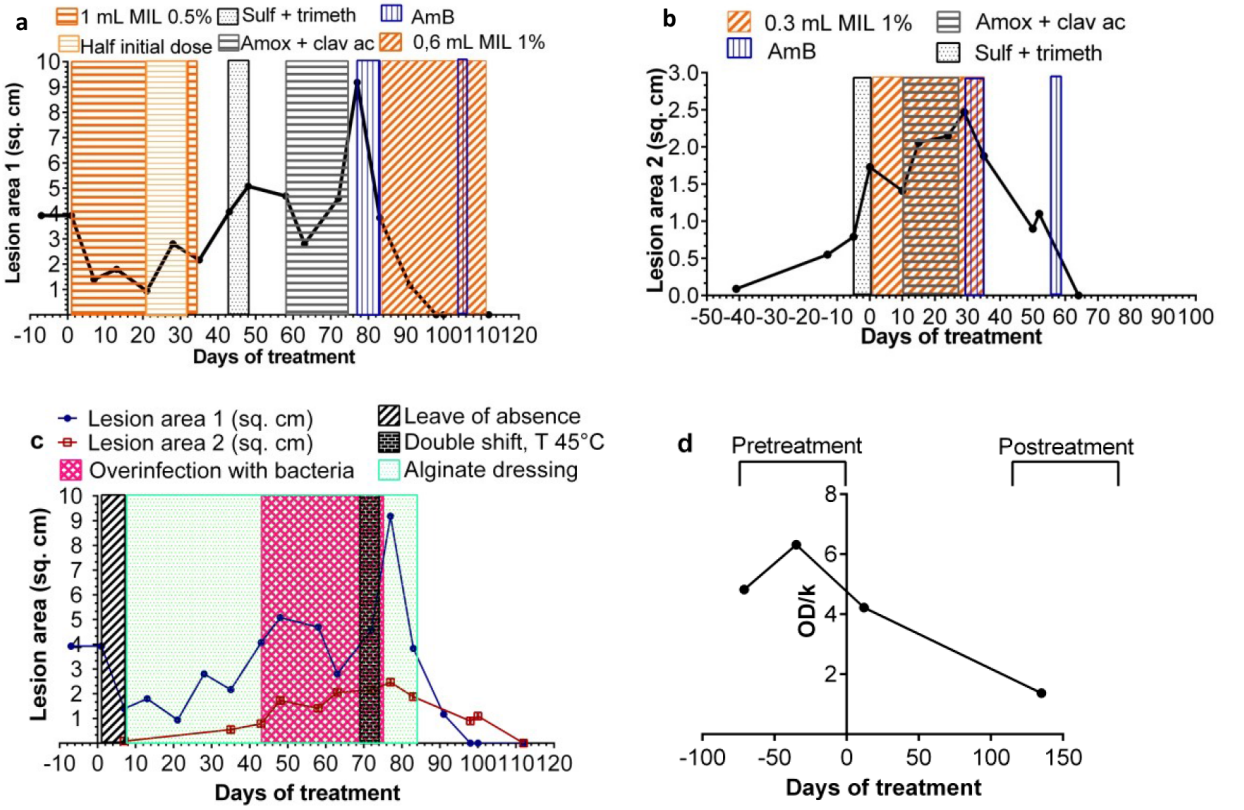

Figure 2. Time evolution of pharmacological treatments, lesion sizes, environmental conditions and anti-Leishmania antibodies. ***a, b)*** *Lesion size and pharmacological treatments vs. time for Lesions 1 and 2, respectively. Sulf = Sulfamethoxazole 800 mg; trimeth = Trimethoprim 160 mg (Bactrim Forte®) (twice a day). Amox = Amoxicillin 875 mg; Clav ac =Clavulanic acid 125 mg (once a day).* ***c)*** *Lesions 1 and 2 size evolution and environmental/secondary conditions.* ***d)*** *Absorbance values/cutoff (OD/k) correlated to anti-Leishmania IgG levels vs. time. In* ***a****,* ***c****, and* ***d****, day 1 of treatment corresponds to the first day of treatment of Lesion 1. In* ***b****, day 1 corresponds to the first day of treatment of Lesion 2.*

Lesion 2

Treatment of Lesion 2 started on day 48, counting from the initiation of treatment of Lesion 1. We could not start it earlier due to a lack of access to MIL. At this point, Lesion 2 had a thick border and was ulcerated and necrotic (Figure 4a and Figure 3). Lesion 2 was treated with 0.3 mL of MIL 1% w/v gel twice a day for 35 days. On day 30, the area peaked, but 80% of the lesion showed granulation tissue and no erythema, lowering the severity score to 2 (Figs. 3 and 4d). The lesion area started to decrease on day 36 (Figure 2b). On day 53, the lesion was completely re-epithelialized (Figure 4f and Figure 3).

	Score
Ulcer + necrosis	6
Flattened edge + inflammation	5
Flattened edge (80% erythema)	4
Flattened edge (20% erythema)	3
Cure tendency (80% granulation tissue)	2
Cure tendency (20% granulation tissue)	1
Cicatrization	0

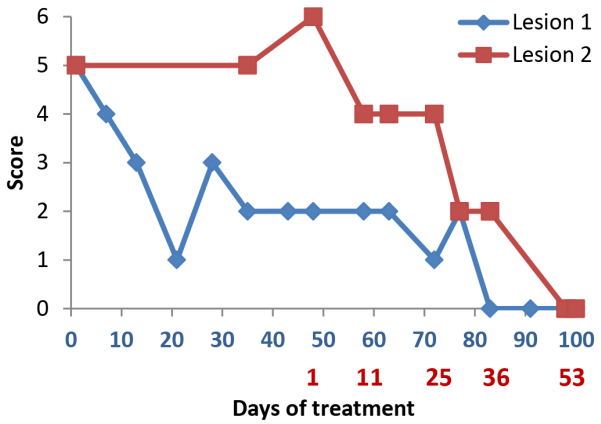

Figure 3. *Severity score of lesions 1 and 2 during treatment. Blue is data for Lesion 1; red is data for Lesion 2.*

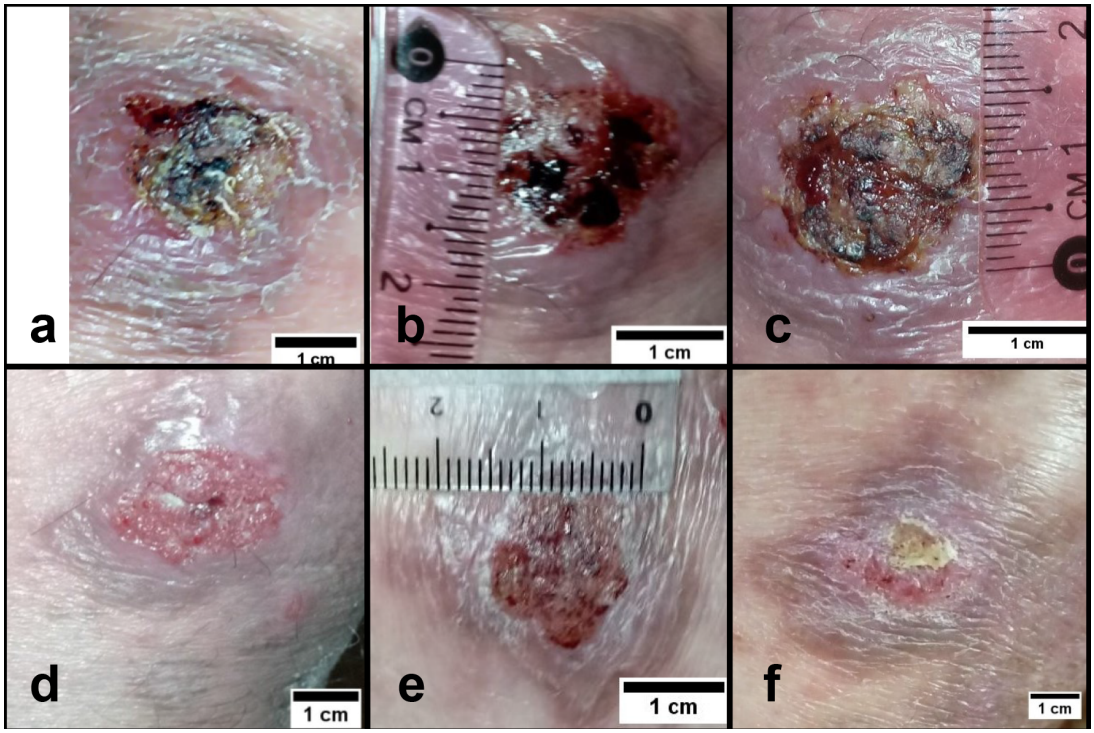

Figure 4. Photographs of Lesion 2. a) day 1 (note the ulcer with necrotic center); b) day 16 (border flattened, erythema); c) day 25 (lesion stable, scab); d) day 30 (80% of area covered by granulation tissue); e) day 36 (scab) and f) day 53 of treatment (re-epithelialization).

Correlation between lesion areas and environmental/secondary conditions

Figure 2c shows the correlation between lesion areas and environmental/secondary conditions. The patient is a rural worker in Orán, Salta, in northern Argentina. Orán has a humid subtropical climate with a dry winter season. His usual working day consists of 10 hours of physical labor in the open, exposed to dirt, insects, sun, and, during the spring and summer, often extreme heat. The treatment took place during the months of September through December (spring-summer). At the start of treatment, the patient took a one-week

leave from work, stayed at home, rested, and applied the gel without occluding the wound. As can be observed in Figure 1, Figure 2c, and Figure 3, during these first days of treatment, Lesion 1 improved considerably, with a 65% decrease in area and a drop of 40% in the severity score. On day 8, the patient returned to work, and from that day on, 6 x 7 cm sterile self-adhesive alginate dressings were used to cover the lesion. On the other hand, during days 68-76 of treatment, the patient was compelled to work double shifts (14-16 hours per day) in temperatures that reached 45 °C. The extreme heat produced profuse sweating, which in turn made the protecting alginate dressing come off during the day. This may have been a factor in the over-infection that took place at this time. Figure 2c shows how the Lesion 1 area increased from 2.8 cm^2 to 9.2 cm^2 (3.3x increase) during this period, even though the patient was undergoing treatment with antibiotics (Sulfamethoxazole + Trimethoprim). Figure 1g-h and the severity score (changing from 1 to 2) also illustrate this episode. Lesion 2 suffered a 4% increase in area but no change in severity score during this period, probably because it was under treatment with MIL gel.

In summary, rest produced a strong positive impact, while working long hours under extreme heat contributed to over-infection and worsening of the wound.

Anti-*Leishmania* IgG in serum samples

As shown in Figure 2d, the amount of anti-*Leishmania* IgG in the patient's serum decreased 4.8 times during the treatment, from an OD/k value of 6.3 (pre-treatment) to an OD/k of 1.3 (day 35 after finishing treatment). This was correlated with a good clinical response. However, it is important to mention that we did not observe seroconversion (OD/k < 0.9). When the treatment is effective, seroconversion may occur between 6 months and one year after the treatment has ended. Therefore, it is important for the clinical and serological follow-up of the patient during this period (Marco JD, unpublished data).

Leishmania braziliensis was the causal agent

The sequences obtained from the sequencing service were compared with those obtained from GenBank in an alignment constructed using MEGA 11 software. The NJ tree was constructed using the MLSTest software. The parasites isolated from Lesion 1 were identified as *L. (V.) braziliensis*. This is the predominant causal agent of CL in Argentina.

The isolated parasite had a low susceptibility to Glucantime and Amphotericin B.

The susceptibility of parasites isolated from Lesion 1 before MIL treatment and after Glucantime and AmB failure was evaluated *in vitro* in a macrophage-amastigote model. After 48 h of incubation with the anti-leishmanial drugs, parasite growth was inhibited significantly ($P < 0.05$) only at the highest concentration of drugs tested: Glucantime (480 µg/mL), AmB (0.35 µg/mL) and MIL (4 µM). The IC50 calculated from these essays were of 509.4 µg/mL for Glucantime and 0.45 ug/mL for AmB, being these values ten times higher than those reported in the literature for the reference strain of *Leishmania braziliensis* (MHOM/BR/75/M2903) [25]. On the other hand, the IC50 = 6.5 µM calculated for MIL was in the range reported recently (3.31 to > 35 µM) for *Leishmania braziliensis* [11]. This susceptibility to MIL is concordant with the good clinical response to MIL observed in the patient.

The development of drug resistance in *Leishmania* is an increasing concern and a key factor for treatment failure. In our case, the parasites were isolated after the patient had already been treated with Glucantime and AmB several times during a three-year period. Therefore, the high IC50 of Glucantime and AmB measured in the isolated parasites could mean two things: either the parasite was already resistant to these drugs at the time of infection, or it became resistant due to the multiple treatments the patient underwent with these drugs. Unfortunately, we did not have access to the samples taken from the patient's lesions at the first diagnosis and so we cannot distinguish between these two possibilities.

Conclusions

The patient improved with the topical treatment but only achieved a complete cure with a combination of topical MIL and intravenous AmB. MIL reduced the area on the lesions and, in combination with conventional therapy with AmB, allowed the clinical cure with a short treatment with this highly toxic drug. The proper dressing of the wounds and the possibility of rest from hard physical labor and extreme heat stand out as extremely important for the good outcome of the treatment. A clinical study in a larger number of patients will help elucidate the efficacy of the MIL gel. Topical MIL is a promising therapeutic agent that needs further development.

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Conflict of interest

The authors declare no conflict of interest. For a signed statement, please contact the journal office at editor@precisionnanomedicine.com.

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