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Effect of topical berberine in murine cutaneous leishmaniasis lesions

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Objectives: More effective topical treatments remain an unmet need for the localized forms of cutaneous leishmaniasis (CL). The aim of this study was to evaluate the efficacy and safety of a topical berberine cream in BALB/c mice infected with *Leishmania major* parasites.

Methods: A cream containing 0.5% berberine-β-glycerophosphate salt and 2.5% menthol was prepared. Its physicochemical and stability properties were determined. The cream was evaluated for its capacity to reduce lesion size and parasitic load as well as to promote wound healing after twice-a-day administration for 35 days. Clinical biochemical profile was used for estimating off-target effects. *In vitro* time-to-kill curves in *L. major*-infected macrophages and skin and plasma pharmacokinetics were determined, aiming to establish pharmacokinetic/pharmacodynamic relationships.

Results: The cream was stable at 40°C for 3 months and at 4°C for at least 8 months. It was able to halt lesion progression in all treated mice. At the end of treatment, parasite load in the skin was reduced by 99.9% (4 log) and genes involved in the wound healing process were up-regulated compared with untreated mice.
The observed effects were higher than expected from *in vitro* time-to-kill kinetic and plasma berberine concentrations, which ranged between 0.07 and 0.22 μM.

Conclusions: The twice-a-day administration of a topical berberine cream was safe, able to stop parasite progression and improved the appearance of skin CL lesions. The relationship between drug plasma levels and *in vivo* effect was unclear.

Introduction

Leishmaniasis is considered a major public health problem because 1 billion individuals are at risk of infection worldwide. Among the different forms of leishmaniasis, cutaneous leishmaniasis (CL) is the most common type and its prevalence has almost doubled from 2.1 million cases in 2002 to nearly 4 million cases in 2015.[1] For decades, pentavalent antimony compounds, administered IV or intralesionally, or topical paromomycin have been the first-line treatment for CL.[2] However, variation in the clinical response has been a persistent problem over the past 50 years.[3] With these issues, and according to the WHO, the search for better topical treatments of CL is a priority for their multiple advantages.[4]

Natural products have played a significant role in the drug discovery process throughout the last century. Among them, berberine chloride [1,8,13α-tetra-hydro-9,10-demethoxy-2,3-(me-thyl-ene-dioxy)-berberium chloride], an isoquinoline alkaloid,

has demonstrated pharmacological activity against several diseases, including leishmaniasis.[5,6] Moreover, it is currently being tested in Phase IV clinical trials for hyperglycaemia and metabolic syndrome and it is approved as an over-the-counter (OTC) drug to treat gastrointestinal infections in China.[7] This status may greatly accelerate its use for other therapeutic applications (therapeutic switching), especially for topical use.

The purpose of this work was assessment of the efficacy of berberine by the topical route in a murine model of CL, encouraged by the following attributes: (i) berberine has shown high intrinsic antileishmanial activity (EC_{50} of 1 μM against *Leishmania donovani* intracellular amastigotes) and excellent selectivity index (SI) of >125 *in vitro*;[8,9] (ii) berberine has anti-inflammatory activity[6] and immunomodulatory properties, mainly mediated by the mitogen-activated protein kinase (MAPK) pathway.[8] Thus, its antileishmanial and anti-inflammatory dual effect could favour the healing of CL lesions without scarring; (iii) the

low molecular weight (around 300 Da) and slightly hydrophilic logP of berberine[10] should not impede its permeation through damaged skin in CL lesions,[11] in which the stratum corneum is partially removed;[12] (iv) berberine suffers rapid hepatic clearance[13] that can avoid systemic accumulation and side effects upon absorption from skin; and (v) berberine has a low cost. The terpene menthol was incorporated in the topical formulation at 2.5%, a concentration that, despite needing precaution,[14] can be used. Furthermore, the antileishmanial activity of this terpenoid[15] could join that of berberine. In addition to this effect, the role of menthol as a permeation enhancer of berberine has been previously reported.[16] Although infection increased the skin permeability,[11] such a type of compound could still be needed in a formulation,[17] as infected macrophages are deeply located in the dermis, surrounded by granulomatous tissue.

Overall, we evaluated berberine efficacy in *Leishmania major*-infected BALB/c mice in terms of both parasite clearance and inflammatory response modulation. Skin and plasma berberine pharmacokinetics (PK) were also determined to establish PK/pharmacodynamic (PK/PD) relationships.

Materials and methods

In vitro time-to-kill curves, cytotoxicity and berberine-β-glycerophosphate/menthol combination studies

Toxicity of berberine-β-glycerophosphate and menthol was assessed in bone marrow-derived macrophages (BMDMs) grown in DMEM by the MTT assay, after 48 h. The activity of berberine-β-glycerophosphate and menthol, either alone or in combination, was then evaluated against intracellular *L. major* amastigotes at different incubation times (24, 48 and 72 h) by the back transformation assay (BTA), as previously reported,[18] using a ratio of 10:1 (parasites:macrophages) and allowing parasite infection overnight. The effective or cytotoxic concentration values (EC_{50}, EC_{90} and CC_{50}) were obtained by fitting the data to a dose–inhibition sigmoid curve using GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). SI was calculated as the ratio between cytotoxicity (CC_{50}) against BMDM and activity (EC_{50}) against *Leishmania* amastigotes. In order to evaluate whether menthol had no interaction, synergy or antagonism in combination with berberine-β-glycerophosphate, an FIC index (FICI) was calculated after 48 h of treatment.[19] See the Supplementary methods for more details (*BTA* and *Drug combination studies* sections), available as Supplementary data at *JAC* Online.

Berberine protein binding in the *in vitro* assay medium (DMEM containing 10% heat-inactivated FBS) was estimated by the ultracentrifugation technique, as previously described[20] and detailed in the Supplementary methods (*In vitro determination of berberine binding to plasma and culture medium proteins* section). This percentage was used to determine the fEC_{50} and fEC_{90} (*in vitro* EC_{50} and EC_{90} against *Leishmania* corrected for protein binding), on the basis of the obtained EC_{50} and EC_{90} values.

Preparation, characterization and stability of berberine-β-glycerophosphate cream

To obtain berberine-β-glycerophosphate cream, 12 g of the oily phase containing cetyl alcohol (1.5%), stearic acid (2.5%), solid paraffin (5%), liquid paraffin (7.5%), glyceryl monostearate (11%) and menthol (2.5%) was melted in a mortar at 70°C. Water (enough for 40 g), berberine-β-glycerophosphate salt (0.5%), Polysorbate® 20 (2.5%) and preservatives [Nipagin™ (0.16%) and EDTA (0.07%)] were then heated at 70°C and poured onto the oily phase under agitation until the mixture was cooled. Cream without berberine-β-glycerophosphate was also

prepared, substituting the amount of salt by water, and identified as vehicle. Their physicochemical properties were evaluated and stability studies were carried out. More details are provided in the Supplementary methods (*Characterization and stability of berberine-β-glycerophosphate cream* section).

Animals

This study was conducted in BALB/c mice (Harlan, Spain) housed in groups of five in plastic cages under controlled environmental conditions (12:12 h light/dark cycle and $22 \pm 2°C$), according to ethical standards approved by the Animal Ethics Committee of the University of Navarra, in strict accordance with the European legislation in animal experiments (protocol number 100-19).

Ex vivo permeation and penetration studies in healthy mouse skin

Permeation studies under infinite conditions were carried out in freshly excised mouse skin according to the Organisation for Economic Cooperation and Development (OECD) guideline 428[21] during 24 h using Franz diffusion cells. The receptor compartments were filled with a PBS solution, enough to ensure sink conditions. Full-thickness excised female mouse skin pieces were placed between the donor and receptor compartments. Receptor fluid samples were taken at determined times (0.5, 1, 2, 4, 6, 8, 10 and 24 h). Berberine in the receptor compartment and in the skin was quantified using HPLC-MS/MS. The flux of drug permeated (*Jss*, μg/cm²·h), lag time (h) and permeability coefficient (*Kp*, cm/h) were then calculated. More details are provided in the Supplementary methods (*Ex vivo permeation studies in healthy mouse skin* section).

In vivo efficacy of berberine-β-glycerophosphate cream in L. major-infected BALB/c mice

After 2 weeks of infection (lesion size around 6 mm²), mice ($n = 5–6$) were left untreated or topically treated with berberine-β-glycerophosphate and its vehicle. Mice received 7.5 mg/kg of berberine-β-glycerophosphate twice a day (15 mg/kg total dose) during 35 consecutive days. Lesions were measured every 4 days with a digital calliper. Three days after the end of treatment, parasite load in popliteal lymph nodes (LNs) and skin lesions was quantified after DNA extraction by quantitative RT–PCR (qRT–PCR). Cytokine expression in skin lesions was also assessed at the end of treatment by qRT–PCR, after RNA extraction. More details are provided in the Supplementary methods (*DNA extraction and parasite quantification* and *RNA extraction and cytokine expression* sections and Table S1).

PK and dermatokinetic (DK) studies during treatment

Blood from non-infected and *L. major*-infected mice ($n = 6$) was collected from the submandibular plexus of mice at determined timepoints after the first daily application on Days 1 and 10. At the same timepoints on Day 1, lesions were carefully washed with methanol (to remove the remaining formulation) and berberine accumulation in the skin lesions was analysed by HPLC-MS/MS. Also, blood was collected on Days 3, 7, 13, 18, 22, 30 and 35. For estimation of PK parameters, healthy mice were injected IV with 7.5 mg/kg berberine (previously dissolved in type I water with 5% glucose at 1 mg/mL) and blood samples were collected at 0.25, 0.5, 1, 4 and 24 h. Plasma concentrations were obtained by centrifugation of blood at 6000 g for 10 min and berberine was quantified by HPLC-MS/MS. PK parameters were calculated using non-compartmental analysis (NCA) and the Excel PKSolver program.[22] More details are provided in the Supplementary methods (*Berberine extraction from plasma and skin samples* and *Quantification of berberine by HPLC-MS/MS* sections).

Berberine binding to plasma proteins was determined after incubating different concentrations of berberine diluted in mouse plasma (from 50 to 1000 ng/mL), using the same procedure described for determining the drug binding in cell culture medium and detailed in the Supplementary methods (*In vitro determination of berberine binding to plasma and culture medium proteins* section).

Histological analysis and immunohistochemistry

At the end of treatments, skin fragments were formalin-fixed, paraffin-embedded, cut in 3 μm thick sections and stained with haematoxylin and eosin (HE). Immunohistochemistry for NIMP-R14 (neutrophils), F4/80 (macrophages) and CD3 (lymphocytes) was also carried out. Digital images were scanned using a digital microscope system (Aperio ScanScope CS2, Leica Biosystems). For immunohistochemistry analysis, the percentage of area stained in each image was then quantified by Fiji 2.0. software. More details are provided in the Supplementary methods (*Immunohistochemistry studies* section).

Biochemical analysis after topical berberine-β-glycerophosphate cream administration: in vivo toxicity studies

Biochemical analysis was performed in non-infected and *L. major*-infected mice. At the end of treatment, blood samples were collected from the submandibular vein in order to evaluate renal and liver toxicity. ALT, AST, creatinine (CRE), alkaline phosphatase (ALP), glucose, total cholesterol (CHO), HDL cholesterol (HDL), LDL cholesterol (LDL), urea (BUN) and triglyceride (TRIG) concentrations were measured in serum in a Cobas® biochemistry analyser (Roche). Non-treated mice were bled for comparison.

Statistical analysis

Statistical analyses between three groups were performed by Kruskal–Wallis (non-parametric) followed by Dunn's multiple comparisons tests. Differences between two groups were analysed by a non-parametric Mann–Whitney test or by parametric *t*-test, depending on their Shapiro–Wilk normality test. GraphPad Prism7 was used to perform the analysis. Significance was established for *P* values of <0.05.

Results

In vitro time-to-kill curves, cytotoxicity and berberine-β-glycerophosphate/menthol combination studies

As presented in Table 1, berberine-β-glycerophosphate showed high activity against *L. major* amastigotes after 48 h of treatment (EC_{50} and EC_{90} of 0.07 and 0.22 μM, respectively) and low cytotoxicity (CC_{50} of 125.3 μM), as an SI of 1790 was determined.

Moreover, as several terpenoids have demonstrated antileishmanial activity,[15] the activity and toxicity of menthol either alone or in combination with berberine-β-glycerophosphate was calculated to determine whether some interaction between the two compounds occurred. After 48 h incubation time, menthol showed low antileishmanial activity (EC_{50} and EC_{90} of 80.8 and 783.3 μM, respectively) and an SI value of 6.9 (Table 1). Although an FICI value of 0.95 was found between berberine-β-glycerophosphate and menthol, which indicated no interaction between the two compounds (Table 1), lower EC_{50} values were calculated in the dose–response curves of berberine-β-glycerophosphate when menthol (10 or 50 μM) was added (Figure S1).

Furthermore, as seen in Figure 1 and Table S2, berberine-β-glycerophosphate reduced amastigote viability in a dose- and time-dependent manner, with lower EC_{50} and EC_{90} values at longer times of treatment. In fact, EC_{50} and EC_{90} values of 0.02 and 0.12 μM, respectively, were obtained for berberine-β-glycerophosphate after 72 h of treatment (Table S2), compared with values of 0.56 and 1.52 μM after 24 h. Moreover, 0.01 and 0.05 μM concentrations of berberine-β-glycerophosphate were unable to achieve maximal amastigote reduction (Figure 1a) after 72 h. Concentrations of 0.1, 0.5 and 1 μM produced complete removal of parasites at long incubation times (72 h). In contrast, 2 μM or higher concentrations of berberine-β-glycerophosphate exhibited maximal activity after 24 h. Overall, it seems that a berberine concentration of 2 μM or higher would be required at the target site (dermal macrophages) to achieve maximal and fast effect.

Finally, the obtained values for *in vitro* berberine-β-glycerophosphate activity were corrected according to the extent of drug–protein binding measured in the culture medium (30.7% ± 8.9%), as the activity of a drug is generally accepted to be produced by the unbound drug concentration at the site of action.[23] These values were 0.01 and 0.08 μM for fEC_{50} and fEC_{90}, respectively, after 72 h (Table S2).

Characterization of berberine-β-glycerophosphate cream

Creams without (vehicle) or containing berberine-β-glycerophosphate were characterized in terms of viscosity, spreadability and pH (Table S3). Berberine-β-glycerophosphate cream showed higher viscosity (0.97 versus 1.29 Pa·s), lower spreadability (2.5-fold) and lower pH (6.09 versus 4.81) compared with vehicle. Berberine-β-glycerophosphate cream was stable for 3 months at the three different storage temperatures (4°C, 25°C and 40°C) (Table 2). However, after 8 months at 25°C and 40°C, the cream
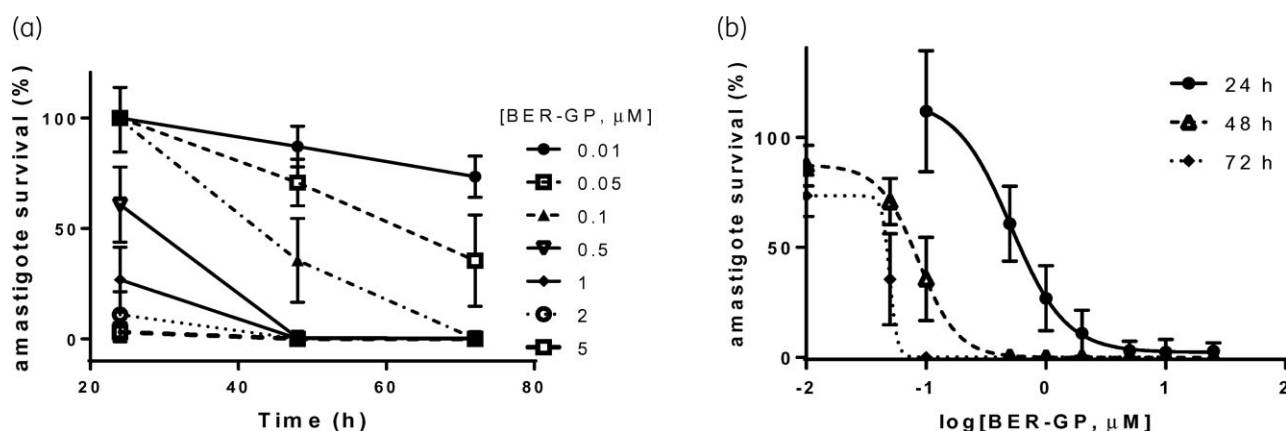
Table 1. *In vitro* activity against *L. major* amastigotes, cytotoxicity in BMDM and SI of berberine-β-glycerophosphate (BER-GP) and menthol (MNT) after 48 h of treatment

	L. major amastigotes		BMDM		
Drug	EC_{50}	EC_{90}	CC_{50}	SI	FICI
BER-GP	0.07 (0.06–0.10)	0.22 (0.16–0.30)	125.3 (107.1–143.4)	1790	0.95
MNT	80.8 (52.5–124.3)	783.3 (429.5–1128.6)	561.3 (338.5–784.1)	6.9	No interaction

EC_{50}, EC_{90} and CC50 data are expressed in μM (95% CI), $n = 3$. FICI value was obtained for amastigotes after 48 h of BER-GP treatment combined with MNT ($n = 3$).

(a)

(b)

Figure 1. (a) Time-to-kill curves for berberine-β-glycerophosphate (BER-GP) at indicated drug concentrations (μM) and (b) sigmoidal fitting of BER-GP salt dose–response at the indicated incubation times. The effect was determined against *L. major* amastigote-infected macrophages and evaluated by the BTA assay. Data (mean ± SD, $n=4$) are expressed as amastigote survival (%), calculated on the basis of untreated infected macrophages.

Table 2. Stability studies for berberine-β-glycerophosphate (BER-GP) cream over a period of 8 months at different storage temperatures

BER-GP cream	3 months			8 months		
	4°C	25°C	40°C	4°C	25°C	40°C
pH	4.70 ± 0.35	3.79 ± 0.30	4.13 ± 0.16	3.71 ± 0.12	3.67 ± 0.20	3.86 ± 0.25
Spreadability (cm)	0.60 ± 0.07	0.50 ± 0.04	0.70 ± 0.14	0.65 ± 0.15	0.70 ± 0.14	0.85 ± 0.08
BER recovery (%)	99.5 ± 2.1	98.2 ± 1.8	98.2 ± 1.8	100.5 ± 2.2	79.2 ± 1.8	73.2 ± 6.2
Colour	Intense yellow	Intense yellow	Intense yellow	Intense yellow	Pale yellow	Pale yellow
Other organoleptic properties	Smooth, soft, characteristic MNT odour			Smooth, soft, characteristic MNT odour		
Phase separation	No evidence	No evidence	No evidence	No evidence	Yes	Yes
Drug precipitation	No evidence	No evidence	No evidence	No evidence	No evidence	No evidence
Gravitational stability	Yes	Yes	Yes	Yes	Yes	Yes

Data expressed as mean ± SD ($n=3$).

showed signs of exudation at 40°C and the recovery of the drug decreased (79.2% and 73.2%, respectively).

Ex vivo permeation studies

Jss (from 72.3 to 98.1 ng/cm²/h) and *Kp* (from 1.4×10^{-5} to 2.0×10^{-5} cm/h) of berberine was slightly increased by adding menthol to the cream (Table 3). Although the role of menthol as a permeation enhancer has been previously described,[24] specifically for this drug in rat skin,[16] the observed effect was modest (Table 3), probably because of the lower menthol concentration used, compared with the study of Patel *et al.*[16] (2.5% versus 12.5%). Moreover, berberine in skin was similar in both groups. The creams had long lag times (3 and 2.5 h), as previously observed with other berberine topical formulations.[25]

In vivo efficacy of topical berberine-β-glycerophosphate cream in L. major-infected BALB/c mice

Two weeks after infection, treatments started. The ability of the treatment to halt the lesion growth started to be observed after 3 days of treatment, although significant differences with untreated control lesions only occurred after 18 days. Lesion

progression stopped in berberine-β-glycerophosphate-treated mice and lesion sizes were significantly smaller than lesions of non-treated mice (7.8-fold; $P<0.001$; Figure 2a and b). Moreover, as seen in Figure 2b, lesions treated with berberine-β-glycerophosphate cream were more superficial and hair started to grow. To determine whether menthol presented some leishmanicidal effect *per se*, mice were also treated with vehicle. No reduction in lesion progression was observed (Figure 2a).

Histological analysis of control (infected, untreated; Figure 2c, left) and berberine-β-glycerophosphate cream-treated skin (Figure 2c, right) confirmed the healing of the lesions, although skin was never completely regenerated and foci of necrosis and inflammation persisted. Accordingly, berberine-β-glycerophosphate cream significantly decreased ($P<0.01$; 99.9%; 4 log) the number of parasites in skin lesions (Figure 2d). Parasite burden in LNs was also significantly reduced ($P<0.05$; 76.6%; 1 log) after topical application of berberine.

Immunohistochemistry and gene expression of CL lesions

Immunohistochemical staining (Figure 3a and b) of skin lesions at the end of berberine-β-glycerophosphate treatment confirmed a

Table 3. *Ex vivo* permeation values obtained for berberine-β-glycerophosphate (BER-GP) creams without or with menthol (MNT) across healthy mouse skin after 24 h

Composition	Jss (ng/cm^2/h)	Kp (cm/h)	Lag time (h)	Permeated BER (ng/cm^2)	BER in skin (ng/mg)
BER-GP (0.5%)	72.3 ± 3.6	1.4×10^{-5}	3	1533.2 ± 789.8	76.7 ± 19.6
BER-GP (0.5%) + MNT (2.5%)	98.1 ± 13.4	2.0×10^{-5}	2.5	2366.4 ± 865.1	72.0 ± 17.1

Results are expressed as mean $\pm$ SD ($n=6$). Comparisons between two groups were made by a parametric *t*-test (no significant results).

significantly lower number of neutrophils (NIMP-R14; $P<0.01$) and a significant reduction in macrophage (F4/80) infiltration ($P<0.05$), consistent with the smaller lesion sizes observed after topical berberine-β-glycerophosphate therapy.

On the other hand, berberine-β-glycerophosphate treatment significantly up-regulated the mRNA expression of (iNOS) ($P<0.01$) as well as COX-2, Arg1, CCL17, CCL22 and IL-10 ($P<0.05$) (Figure 3c). In contrast, CCL2 appeared down-regulated after treatment ($P<0.05$). The higher expression of iNOS indicates that treatment with berberine could activate macrophages to produce nitric oxide (NO), leading to the killing of *Leishmania* parasites. Moreover, the up-regulation of Arg1, CCL17, CCL22 and IL-10 and the down-regulation of CCL2 could be in accordance with wound-healing repair mechanisms.

PK and DK studies

Berberine plasma concentration (C_{trough}) and drug concentration in the total skin homogenates after a single (at Day 1) or multiple doses (at Days 3, 7, 12, 18, 22, 30 and 35) of topical berberine-β-glycerophosphate cream administration is shown in Figure 4 (a and b, respectively). PK and DK parameters obtained using NCA after the first application (Day 1) and at Day 10 are summarized in Table 4.

Either in healthy or infected mice, berberine concentration quantified in skin and plasma was higher at Day 10 (after several administrations) than at Day 1, probably due to the time required to achieve steady state more than drug accumulation, as C_{trough} remained constant from Day 3 to the end of the experiment (Figure 4). As expected and evidenced in Figure 2a, the infection
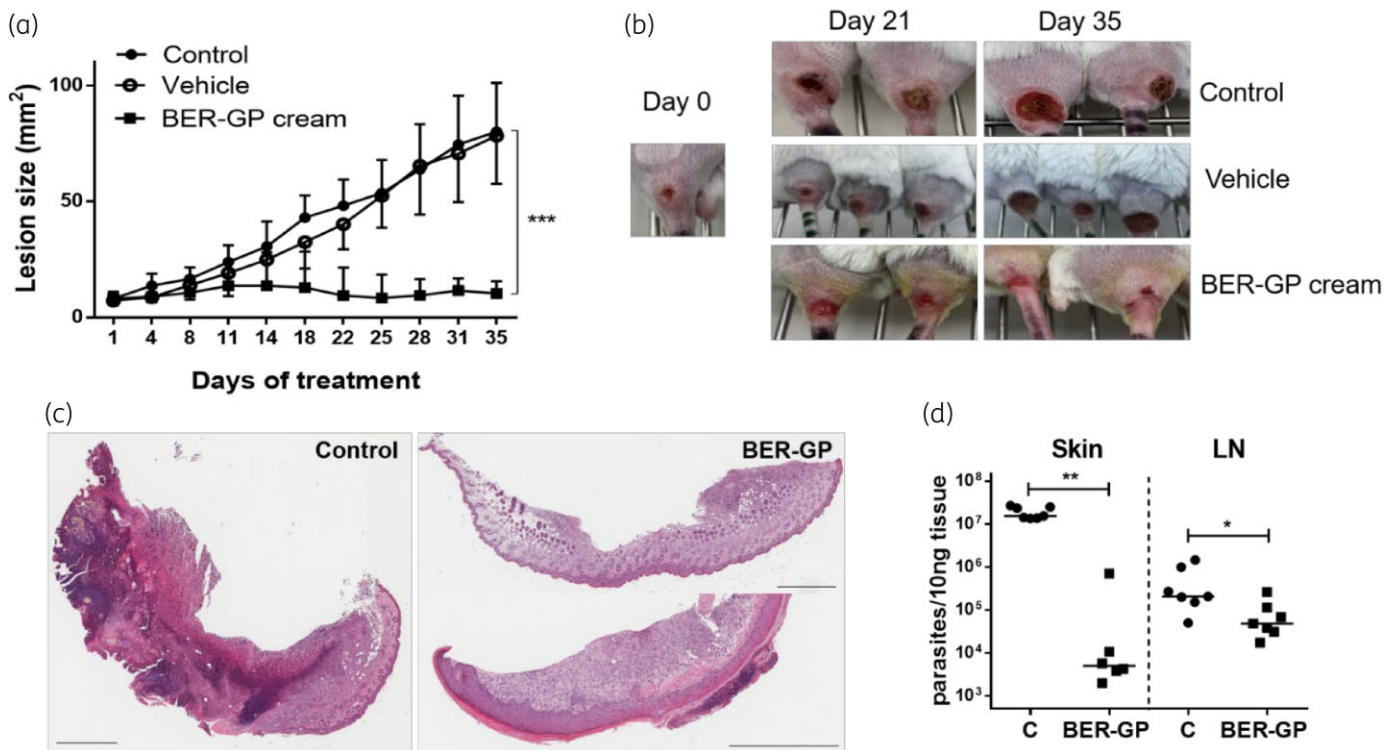

Figure 2. (a and b) Lesion size progression during topical berberine-β-glycerophosphate (BER-GP) cream treatment (control, filled circles; vehicle, open circles; and BER-GP cream, filled squares). (c) Representative images of HE-stained skin sections obtained from a control mouse (infected and non-treated, left) and BER-GP cream treated mice (right). Scale bars: 800 μm. (d) Parasite burden in skin lesions and LNs of *L. major*-infected BALB/c mice after 35 days of topical treatment with BER-GP cream (15 mg/kg daily in two doses, filled squares), compared with non-treated mice (C=control, filled circles). Results are expressed as median ($n=6–11$ per group). Data were analysed by a non-parametric Mann–Whitney test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.
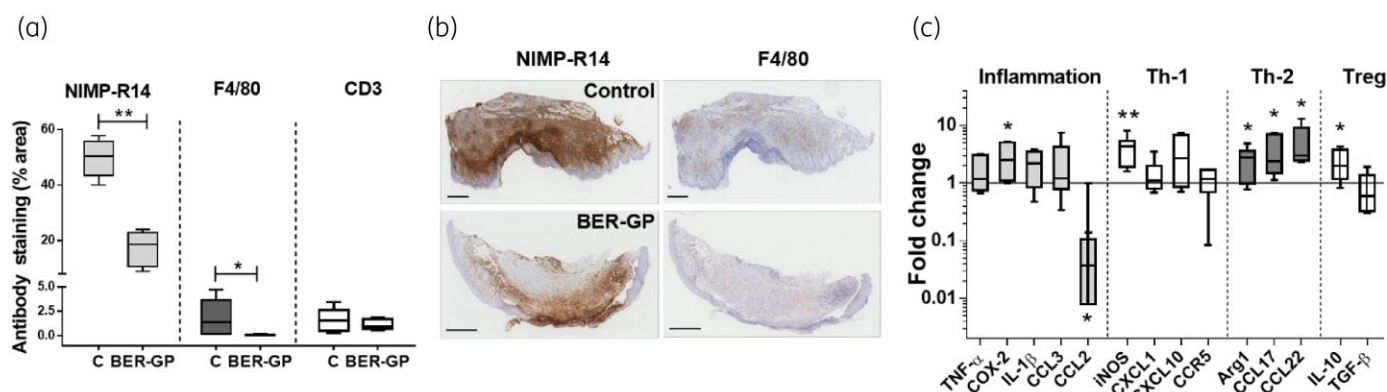
Figure 3. (a) Immunohistochemical analysis in skin lesions of non-treated *L. major*-infected BALB/c mice (C = control), or after 35 days of topical treatment with berberine-β-glycerophosphate (BER-GP) cream for neutrophils (NIMP-14, light grey), macrophages (F4/80, dark grey) and lymphocytes (CD3, white). Boxes represent median (central line) ± 95% CI (upper and lower edges) and whiskers represent minimum and maximum values (*n* = 5 per group). (b) Representative images of skin sections for control (infected untreated mice, top) and mice treated with BER-GP cream (bottom) stained with antibodies against NIMP-R14 (neutrophils) and F4/80 (macrophages). Scale bars: 600 μm. (c) Cytokine expression in skin lesions from *L. major*-infected BALB/c mice after 35 days of topical treatment with BER-GP cream, compared with non-treated mice (C = control), expressed as fold change. Boxes represent median (central line) ± 95% CI (upper and lower edges) and whiskers represent minimum and maximum values (*n* = 5 per group). Data were analysed by a non-parametric Mann–Whitney test. * $P < 0.05$, ** $P < 0.01$. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

seriously disrupted the barrier integrity of skin[11] and 2-fold more berberine was quantified in the plasma of infected mice than in healthy ones (AUC of 448.2 versus 262.8 ng/mL·h, respectively, at Day 10; not significant; Table 4). The drug ratio between the skin and plasma[26] was also changed by the infection, from around 1:2 in healthy versus 1:1 in damaged skin (comparison made once achieved the steady state; $P < 0.001$; Table 4). Berberine-β-glycerophosphate, with a logP value of −0.04 (experimental determination) would have more affinity with infected skin, a more hydrophilic substrate than intact skin, mostly due to stratum corneum removal and inflammation. Accordingly, the infection doubled the $t_{1/2}$ of berberine in the skin (18.0 versus 7.5 h; Table 4).

After topical berberine administration (Day 1), the bioavailability was around 4% in healthy mice and 12% in infected

ones (Table 4). However, these values diminished to around 1% or lower when expressed as percentage of applied dose. This issue highlights rapid liver clearance as the major disadvantage for berberine systemic bioavailability, which is a very helpful property if dermal, but not systemic, drug delivery is intended. The bioavailability of berberine has also been previously described to be much higher after its topical administration than after its oral administration in rats.[27]

The equilibrium of free drug fraction among different body compartments (in this case among plasma and skin, and then inside macrophages) is a common assumption in order to establish PK/PD relationships.[28] Since 74.8% ± 5.0% of berberine binding to mice plasma proteins was calculated, we inferred that the *in vivo* efficacy of berberine with an unbound plasma concentration was 0.02 μM, lower than berberine *in vitro* fEC$_{50}$ after 24 h
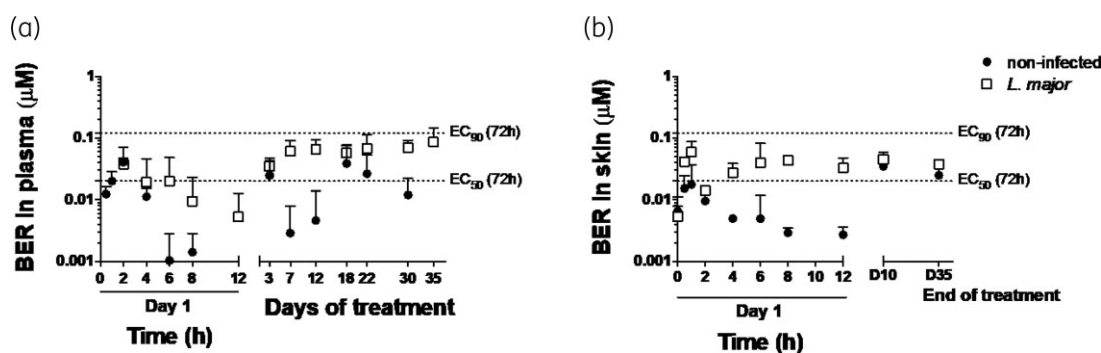

Figure 4. (a) Berberine (BER) plasma concentration at different times (0–8 h) on Day 1 and over the course of the BER-β-glycerophosphate (BER-GP) cream topical treatment in non-infected (filled circles) and *L. major*-infected mice (open squares), measured in blood extracted 12 h after the last daily administration (C$_{trough}$). (b) Skin BER levels at different times (0–12 h) on Day 1, Day 10 (D10) and Day 35 (D35), 12 h after the last treatment. Results are expressed as mean ± SD (*n* = 2–4).

Table 4. PK parameters in non-infected mice after IV administration of a berberine-β-glycerophosphate (BER-GP) solution (7.5 mg/kg) or in blood and skin of non-infected and *L. major*-infected BALB/c mice after a single topical treatment with a BER-GP cream at dose of 7.5 mg/kg (Day 1) or after chronic BER-GP cream administration twice daily during 9 days (Day 10)

| Parameter | IV non-infected | Topical non-infected | | | Topical *L. major* | | |
| | Day 1 | Day 1 | | Day 10 | Day 1 | | Day 10 |
	Plasma	Skin	Plasma	Plasma	Skin	Plasma	Plasma
$t_{1/2}$ (h)	12.9 ± 3.5	7.5 ± 6.1	1.5 ± 0.4	3.5 ± 3.1	18.0 ± 5.3	9.7 ± 6.4	5.6 ± 2.9
T_{max} (h)	0.25	1	2	1	1	2	1
C_{max} (ng/mL)	99.2 ± 36.0	9.7 ± 4.9	17.8 ± 6.3	47.5 ± 29.3	25.3 ± 7.6	14.0 ± 3.1	89.2 ± 62.8
AUC_t (ng·mL·h)	504.6 ± 213.8 (24 h)	27.4 ± 6.5 (12 h)	42.9 ± 16.7 (24 h)	262.8 ± 142.0 (12 h)	154.9 ± 19.4*** (12 h)	85.5 ± 43.6 (24 h)	448.2 ± 253.0 (12 h)
AUC_∞ (ng·mL·h)	976.8 ± 326.7	28.1 ± 3.4	44.2 ± 16.8	328.4 ± 203.3	335.4 ± 189.9**	123.8 ± 66.9	608.7 ± 306.9
F (%)	100		4.5			12.7	

AUC_t, area under the plasma concentration–time curve from time = 0 to time = t; AUC_∞, area under the plasma concentration–time curve from time = 0 to time = infinity; F, bioavailability, calculated as: $(AUC_{topical} \times Dose_{IV})/(AUC_{IV} \times Dose_{topical})$. Data were calculated using NCA and expressed as mean ± SD (n = 3–5). Comparisons between two groups were made by a parametric *t*-test comparing (i) skin of non-infected versus *L. major*-infected mice (** $P < 0.01$, *** $P < 0.001$); (ii) plasma of non-infected versus *L. major*-infected mice at Day 1 (no significant results) and (iii) plasma of non-infected versus *L. major*-infected mice at Day 10 (no significant results). ** $P < 0.01$, *** $P < 0.001$.

(0.39 μM; Table S2) but between the fEC$_{50}$ (0.01 μM) and fEC$_{90}$ (0.08 μM) values estimated at longer incubation times (Table S2 and Figure 4a).

Biochemical analysis after topical berberine-β-glycerophosphate administration: in vivo toxicity studies

Glucose, TRIG and cholesterol (CHO, HDL and LDL) levels, which are normally increased in several metabolic disorders and reduced after berberine administration,[29] did not change at the end of berberine-β-glycerophosphate cream treatment (Table 5), neither in non-infected nor *L. major*-infected mice. Moreover, the levels obtained for the two transaminases (ALT and AST) and ALP were similar in control and treated mice, indicating correct liver function (Table S4). CRE and BUN levels, measuring kidney correct function, were also similar among groups (Table S4). All of these values are within the normal values reported from Charles Rivers for healthy BALB/c mice.[30]

Discussion

This work analysed the efficacy of a topical berberine cream in a murine model of CL produced by *L. major*. At the end of treatment, the smaller lesion size of berberine-treated mice correlated with a decrease of 99.9% (4 log) in the parasite load (Figure 2d), lower inflammatory cell infiltration (especially neutrophils) (Figure 3a and b) and up-regulation of genes involved in the process of skin repair such as IL-10 or Arg-1 (Figure 3c).

In mice models, and more especially in humans,[31] lesion evolution depends on parasite burden but it is also influenced by the inflammatory response.[32] Therefore, the immune response could affect parasite survival and critically determine tissue damage and healing of lesions as well as response to treatment.[33] In fact, in humans, immune mediators (such as TNF-α or granzyme B) and not the parasite are considered as the main factor responsible for tissue damage[34] and there are many efforts addressing the unravelling of immunological pathways able to prevent tissue destruction without affecting parasite load.[35] Drugs with dual leishmanicidal and anti-inflammatory effects would benefit both 'clinical cure' and 'parasite clearance', preventing tissue scarring, residual lesions and future reactivation of the infection. Berberine showed both properties, as both anti-*Leishmania* and anti-inflammatory effects were observed at the end of treatment. However, it cannot be elucidated whether the effect was produced directly or indirectly by the leishmanicidal effect of berberine (the leishmanicidal effect would occur before the healing process), as these determinations were only carried out at the endpoint and not during the course of the treatment.[36] However, berberine is more widely known for its anti-inflammatory properties.[37] In this context, the C57BL/6 mouse strain would be more suitable to evaluate the role of the anti-inflammatory potential of berberine in the course of CL lesions.[38]

PK/PD indices have been established as a very useful tool for optimizing the efficacy and administration schedule of common antimicrobials. However, PK/PD relationships are not well characterized for anti-leishmanial agents[39,40] and far less after their topical application.[41,42] The efficacy should be determined by the drug concentration inside dermal macrophages.[43] An approach

Table 5. Biochemical parameters of non-infected and *L. major*-infected mice after treatment with berberine-β-glycerophosphate (BER-GP) cream compared with untreated control mice

| Parameter | Non-infected | | *L. major* | |
	Control	BER-GP cream	Control	BER-GP cream
TRIG (mg/dL)	114.8 ± 7.4	256.8 ± 87.0	82.5 ± 14.8	100.7 ± 20.7
CHO (mg/dL)	104.4 ± 8.6	130.3 ± 13.6	90.0 ± 9.1	113.1 ± 8.4
HDL (mmol/L)	2.4 ± 0.1	2.3 ± 0.3	1.8 ± 0.1	2.3 ± 0.1
LDL (mmol/L)	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1

Results are expressed as mean ± SD ($n = 5$). Multiple comparisons between groups were made by a non-parametric Kruskal–Wallis test followed by Dunn's multiple comparisons test (no significant results).

to determining this is through the determination of the free drug concentration in the dermis, which requires laborious techniques such as microdialysis.[28,44] In the absence of this information, the free drug plasma concentration can be considered as the best surrogate correlation.[45] There are previous studies confirming this matter in CL.[26,46] However, the observed *in vivo* efficacy of berberine (around 90% parasite reduction in the skin; Figure 2d) was higher than what could be deduced from unbound plasma drug concentration (PK parameter) (C_{trough} at steady state around 0.02 μM) and *in vitro* fEC_{50} obtained even after 72 h incubation time (PD parameter) (0.01 μM; Table S2). In detail, from *in vitro* time-to-kill data, a drug concentration higher than 0.05 μM would be required to achieve 70% parasite clearance (Figure 1a).

This discrepancy between *in vitro* potency and *in vivo* efficacious concentrations could be due to multiple reasons.[47] First, we did not measure the unbound drug, but total drug concentrations were corrected according to the extent of protein binding measured *in vitro*. Second, free plasma concentrations may represent a reliable surrogate for drug exposure inside macrophages, assuming rapid equilibrium between membranes and passive diffusion.[47] Berberine has been described as a substrate for P-gp proteins.[48] Moreover, berberine is a cationic molecule and its intracellular accumulation inside the lysosomes of macrophages could be higher than detected in plasma, as previously described for azithromycin.[49] Third, the *in vivo* berberine activity could involve participation of the immune system, not represented in the *in vitro* studies.[36] Finally, we can also speculate about the contribution of some berberine metabolites (with demonstrated *in vitro* antileishmanial activity, such as oxyberberine, 8-cyanodihydroberberine or tetrahydroberberine)[50] to the effect observed *in vivo*. Berberine anti-diabetic effects were actually observed with plasma concentration of berberine after oral administration far below the concentration required to modulate insulin-signalling pathways *in vitro*.[13,51]

Berberine, as its sulphate salt, has been already tested in an *L. major*-BALB/c mice model.[52] The treatment was not able to stop lesion progression after 10 days of treatment. The possible reasons for this disagreement would be: (i) we evaluated another salt of the drug; (ii) we have included the terpenoid menthol as permeation enhancer, and (iii) we treated the mice for a longer

time. An additional antileishmanial effect of menthol cannot be excluded.

Overall, berberine-β-glycerophosphate salt in a cream containing 2.5% of menthol avoided parasite progression and favoured the lesion healing of *L. major*-infected BALB/c mice. The schedule of topical berberine application twice daily at a dose of 7.5 mg/kg did not show any 'off-target' effects (Table 5 and Table S4) such as lipid lowering or glucose regulation, as currently seen after oral administration of berberine. Pending tasks are to determine the suitable PK driver for berberine efficacy and find the best surrogate marker for it. On the other hand, the data on berberine systemic bioavailability (quite high) after topical administration of the cream (Table 4) allow us to reconsider the need to incorporate menthol as a permeation enhancer in the formulation.

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None to declare.

Supplementary data

Supplementary methods, Tables S1 to S4 and Figure S1 are available as Supplementary data at *JAC* Online.

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