In vivo studies on the antileishmanial activity of buparvaquone and its prodrugs

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Objectives: The efficacy of different formulations of the naphthoquinone buparvaquone and two phosphate prodrugs in in vivo models of both visceral and cutaneous leishmaniasis is described.

Methods: Several topical formulations of buparvaquone containing acceptable excipients were tested in vivo against Leishmania major cutaneous lesions in BALB/c mice. In vivo studies against Leishmania donovani investigated whether the prodrugs had improved efficacy when compared with buparvaquone.

Results: Both a hydrous gel and water-in-oil emulsion of buparvaquone significantly reduced cutaneous parasite burden (P < 0.05, 22 days post-infection) and lesion size, compared with the untreated control (P < 0.0001, 16 days post-infection). The prodrug 3-phosphonooxymethyl-buparvaquone was formulated into an anhydrous gel and this also significantly reduced parasite burden and lesion size (P < 0.0001, 16 days post-infection). Histology confirmed this efficacy. In the visceral model, both prodrugs were significantly more effective at reducing liver parasite burden than the parent drug, buparvaquone. Buparvaquone-3-phosphate was shown to be the most effective antileishmanial (P = 0.0003, 50 mg buparvaquone molar equivalent/kg/day five times), reducing the liver parasite burden by ~34% when compared with the untreated control.

Conclusions: The introduction of a topical formulation, such as buparvaquone (or its prodrug), would be a significant advance for the treatment of simple cutaneous lesions. In particular, the avoidance of the parenteral antimonials would greatly increase patient compliance and reduce treatment costs.

Keywords: leishmaniasis, chemotherapy, cutaneous, visceral naphthoquinones

Introduction

Leishmaniasis is a worldwide disease caused by protozoan parasites of the genus Leishmania, which cause a range of diseases in humans, ranging from disfiguring cutaneous lesions (CLs) to visceral leishmaniasis (VL); the latter is fatal if left untreated. CL is the most common form of leishmaniasis and has an annual incidence of 1–1.5 million cases (90% of these are found in the Old World).1 VL (also known as kala-azar) is found in more than 80 countries in Asia and Africa (Leishmania donovani), Southern Europe (Leishmania infantum) and South America (Leishmania chagasi). Of the 500 000 cases annually, more than 90% occur in Bangladesh, Brazil, Nepal, India (especially Bihar state) and Sudan. It is estimated that India alone accounts for up to 50% of worldwide cases.2 Treatment for VL mainly relies on the parenteral administration of pentavalent antimonials [e.g. 20 mg/kg/day intramuscular (im) or intravenous (iv) sodium stibogluconate for 20 days], which are
associated with many problems (i.e. resistance, toxicity and cost). Recent advances have included the introduction of miltefosine and newer formulations of existing drugs, for example, liposomal amphotericin B.

In CL, the disease is normally localized to the site of infection within dermal macrophages. Typically, papules develop at the site of infection, enlarge to a nodule and progress to ulcerated lesions, which last less than a year. Treatment for CL aims to accelerate healing, minimize scarring and prevent the development of more complex manifestations. Dissemination may occur when certain species migrate to mucosal tissue (i.e. MCL) or multiple cutaneous sites. Systemic therapy is usually warranted in these complex cases. However, for self-limiting forms of CL (such as Leishmania major and Leishmania mexicana) not at risk of more complex manifestations, topical therapy offers a more acceptable form of treatment. Advantages of topical therapy include reduced cost (avoid hospitalization), lower toxicity (target drug to infected tissues) and patient compliance (non-invasive administration). Studies in the Old World show intrasional administration to give superior healing rates when compared with intramuscular antimonials. Currently, there are only two topical formulations, commercially available for the treatment of CL, both of which contain the aminoglycoside, paromomycin, formulated as an ointment. Advantages of topical therapy include reduced cost (avoid hospitalization), lower toxicity (target drug to infected tissues) and patient compliance (non-invasive administration). Studies in the Old World show intrasional administration to give superior healing rates when compared with intramuscular antimonials.

Buparvaquone (BPQ), a hydroxynaphthoquinone shown in Figure 1, is currently marketed as Butalex, an im injection for the treatment of theileriosis in cattle. It was first shown to have antileishmanial activity against L. donovani by Croft et al., where a 62% suppression of hepatic amastigote burden was observed in L. donovani-infected BALB/c mice when BPQ was administered subcutaneously in corn oil (100 mg/kg/day for 5 days). It has several physicochemical properties suitable for topical delivery (low molecular weight, low melting point, etc.). However, it also has low aqueous solubility and high lipophilicity; therefore, the phosphate prodrug approach was investigated in an attempt to increase aqueous solubility and absorption (Table 1). In an earlier study, BPQ and its two phosphate prodrugs (shown in Figure 1), buparvaquone-3-phosphate (BPQ-3-phos) and 3-phosphonoxyethyl-buparvaquone (3-POM-BPQ), were shown to have potent in vitro antileishmanial activity (nanomolar range) against species causing both visceral and cutaneous leishmaniasis. Formulations of BPQ and 3-POM-BPQ were subsequently shown to penetrate both human epidermal and full thickness BALB/c mouse skin in vitro.

The aim of this study was to investigate the in vivo efficacy and toxicity for formulations of buparvaquone and two phosphate prodrugs in animal models for CL and VL. As BPQ was previously shown to have potent in vivo activity in a model for VL, studies were included using the L. donovani BALB/c model to evaluate whether oral delivery of prodrugs had improved efficacy when compared with BPQ.

Table 1. Buparvaquone prodrug physicochemical data

<table>
<thead>
<tr>
<th>Properties</th>
<th>BPQ</th>
<th>BPQ-3-phos</th>
<th>3-POM-BPQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log D</td>
<td>7.02 (pH 3.0)</td>
<td>1.87 (pH 3.0), 1.27 (pH 5.0), 0.47 (pH 7.4)</td>
<td>2.96 (pH 3.0), 1.83 (pH 5.0), 1.16 (pH 7.4)</td>
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<td>pKₐ</td>
<td>5.70</td>
<td>2.68; 6.74</td>
<td>2.28; 6.76</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>ND</td>
<td>121.9–124.0</td>
<td>ND</td>
</tr>
<tr>
<td>Aqueous solubility (mg/mL)</td>
<td>&lt;0.03 μg/mL</td>
<td>&gt;3.5 (pH 3.0, pH 5.0, pH 7.4)</td>
<td>&gt;3.5 (pH 3.0, pH 5.0, pH 7.4)</td>
</tr>
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ND, not determined.
Adapted from Mäntylä et al.
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Materials and methods

Materials

BPQ and Pentostam (sodium stibogluconate) were gifts from GlaxoSmithKline, UK. The BPQ prodrugs, 3-POM-BPQ and BPQ-3-phos, were synthesized at Kuopio University, Finland.14 All chemicals and solvents were of the highest grade available. The formulations were prepared using only FDA-approved or GRAS-listed excipients,16 with the exception of the novel silicones, which were supplied by Dow Corning (Converting, UK). Isopropyl myristate (IPM), polyethylene glycols (PEG300/400), mineral oil, glycerin, paromomycin (as sulphate) and hydroxypropyl-β-cyclodextrin (HP-β-CD) were purchased from Sigma, Poole, UK. Klucel HF Pharm (hydroxypropyl cellulose) was obtained from Hercules, Hopewell, USA. Ethanol and sodium chloride were purchased from BDH, Poole, UK. Carbopol ETD2020 was obtained from Noveon, Cleveland, USA. Cetomacrogol 1000 (Rhodasurf) was obtained from Rhodia, Widnes, UK. Cetostearyl alcohol was obtained from Paroxite, London, UK. White soft paraffin (Vaseline) was obtained from Lever Faberge, London, UK.

Animals

BALB/c (female, 6–8 weeks) mice were obtained from Harlan Sera-Lab, Loughborough, UK and weighed ~20 g each at the time of infection. A standard rodent diet (SDS R and M No. 1 expanded) and de-ionized water were supplied with de lium. Female golden hamsters (Mesocricetus auratus), used for the routine passage of L. donovani, were obtained from Charles River Ltd, Margate, UK. All animals used in these studies were ‘specific pathogen free’. Experiments were conducted under licence in accordance with UK Home Office approval (Project Licence 70/04779).

Parasites

Promastigotes of L. major (MHOM/SA/85/JISH118) were taken from liquid nitrogen stabiles and cultured in Schneider’s Drosophila medium (GibcoBRL, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Harlan Sera-Lab, Crawley, UK). They were maintained at 26°C. L. donovani amastigotes were isolated from the female golden hamster spleen (M. auratus).

In vivo VL leishmaniasis model

The in vivo model for VL has been described previously.17 Briefly, BALB/c mice were infected with L. donovani J118 promastigotes. These were shown to contain ~15% metacyclic promastigotes by PNA agglutination. Lesions were slow to appear and topical treatment was started 10 days post-infection and the study was completed on the 25th day post-infection. Approximately 30–50 mg of each formulation was applied daily on each mouse. Tissue samples were taken at necropsy for real-time PCR analysis to estimate parasite burden.

The second in vivo study compared both BPQ and 3-POM-BPQ formulations. These included a 3-POM-BPQ anhydrous gel (0.70% w/w BPQ and 4.95% w/w 3-POM-BPQ); BPQ w/o emulsion A (0.40% w/w); BPQ w/o emulsion B (0.46% w/w); BPQ hydrous gel (0.21% w/w) and 3-POM-BPQ hydrous gel (0.11% w/w BPQ and 0.24% 3-POM-BPQ). Mice were infected with ~2.0 × 10⁸ L. major J118 promastigotes, including ~10% metacyclics as shown by PNA agglutination. Lesions were slow to appear and topical treatment was started 23 days post-infection. The study was completed on the 40th day post-infection. The mice had received a total of 13 doses, which was calculated as ~70–80 mg of formulation per mouse as a daily dose. Tissue samples were taken at necropsy for real-time PCR analysis to estimate parasite burden.

The third study again compared BPQ and 3-POM-BPQ formulations. These included BPQ hydrous gel (0.05% w/w); 3-POM-BPQ anhydrous gel (2.0% w/w BPQ and 5.0% w/w 3-POM-BPQ); BPQ w/o emulsion A (0.5% w/w) and BPQ in IPM (0.5% w/w). Mice were infected with ~2.0 × 10⁹ L. major J118 promastigotes (10 mice per group) and topical treatment was started in this study 3 days after infection. The treated mice received a total of 20 doses. At 16 and 22 days post-infection, one mouse was dosed once daily by oral gavage (maximum volume 0.4 mL) for 5 days, except for the Pentostam group, which was dosed subcutaneously in 0.2 mL.

Compound efficacy was assessed by determining microscopically the reduction in amastigote burden within the liver. Impression smears were taken 14 days post-infection (7 days after the start of treatment). Slides were examined by light microscopy, using ×1000 oil immersion. The number of amastigotes per 500 cell nuclei was counted in both treated and untreated mice. Body weights were determined pre- and post-treatment as a gross indicator of toxicity.

In vivo CL leishmaniasis model

The in vivo model for CL has been described previously.21 Briefly, mice were infected subcutaneously (0.2 mL) with 10⁶–10⁷ late stage L. major JISH118 promastigotes. Peanut agglutinin (PNA) was used to determine the number of metacyclic promastigotes (‘ Infective inoculum’).22

The paromomycin–urea ointment was included as this is one of the two topical formulations clinically available for CL.2,7,19 Cutaneous lesions were measured weekly using digital callipers (Jencons™) and compared with the untreated control to evaluate therapy. Topical formulations were applied only to the lesions. Body weights were determined for each individual mouse throughout the study as a gross indicator of toxicity. Signs of skin irritation were graded according to the OECD Guidelines for Testing of Chemicals No. 404 (adopted 24 April 2002); a scale used by both UK and US regulatory authorities. The scale is graded 1–4 for both erythema/eschar and oedema formation, with 0 representing ‘none’ and 4 representing ‘severe’.

The first in vivo study was to investigate the toxicity/efficacy of buparvaquone formulations. All the formulations had been tested previously in vitro and shown to penetrate full thickness BALB/c skin.15 The formulations included the paromomycin–urea ointment, anhydrous gel (0.74% w/w), hydrous (0.08% w/w) and w/w emulsion (0.89% w/w). Mice were infected with ~2.2 × 10⁶ L. major JISH118 promastigotes. These were shown to contain ~15% metacyclic promastigotes by PNA agglutination. Topical treatment was started 10 days post-infection and the study was completed on the 25th day post-infection. Approximately 30–50 mg of each formulation was applied daily on each mouse. Tissue samples were taken at necropsy for real-time PCR analysis to estimate parasite burden.
sacrificed per group and parasite burden was estimated by both real-time PCR and the limiting dilution assay (LDA) method. Results were expressed as number of parasites per milligram tissue. At necropsy, one mouse per group was used for histopathology.

Real-time PCR
Parasite burden (from dermal scrapings) was determined using real-time PCR. 24 Real-time PCR analysis was carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the Quantitect SYBR Green PCR kit (Qiagen, UK; Cat 204143). Extraction of genomic DNA was carried out using a DNeasy tissue kit (Qiagen; Cat 69504). Three lesion samples of ~10–20 mg were taken from each mouse. As a requirement for the analysis, Picogreen (Qiagen) was used to quantify DNA in extracts. On the basis of a 25 μL volume for the Quantitect SYBR Green reaction, all samples tested were confirmed to be <500 ng per reaction. Tissue homogenates from naive mice were spiked with known amounts of cultured L. major promastigotes (10-fold serial dilutions), extracted in duplicate and assayed. A standard curve was generated and compared with the same quantity of promastigotes in the absence of skin.

Limiting dilution assays
LDAs were carried out to estimate the number of viable L. major parasites within infected dermal tissue, as described previously. 25,26 Briefly, serial 10-fold dilutions were prepared from homogenized dermal scrapings and the dilutions were placed in 96-well plates (Becton–Dickinson, Franklin Lakes, USA). Following ~2 weeks incubation at 26°C, the plates were read to score the number of positive wells (containing one or more promastigotes). Estimates of parasite frequency and hence burden were carried out using the single-hit Poisson model equation by χ² minimization. 27

Formulations
The BPQ anhydrous gels consisted of ~80% w/w ST-Elastomer 10, 19% w/w cyclomethicone 5-NF and 1% w/w IPM. The 3-POM-BPQ anhydrous gels consisted of ~40% v/v PEG300 and 60% v/v ethanol for gel A and 30% v/v PG and 70% v/v ethanol for gel B. Klucel HF Pharm was used as a gelling agent (~2% w/w). The hydrous gels consisted of ~10% w/w IPM and 90% w/w carbopol ETD2020 mucilage (3% w/v carbopol in water). A 10% w/v sodium hydroxide solution was used to neutralize the final formulation.

The BPQ w/o emulsion (A) consisted of ~19% w/v mineral oil, 6% w/w IPM, 2% w/w emulsifier 10, 2% w/w sodium chloride and 71% w/w water. The w/o emulsion (B) consisted of ~10% w/w cyclomethicone 5NF, 10% w/w IPM, 5% w/w dimethiconol 20, 2% w/w silky wax 10, 2% w/w emulsifier 10, 1% w/w sodium chloride, 3% w/w glycerin and 67% w/w water. The BPQ o/w emulsion consisted of ~75% w/v carbopol ETD2020 mucilage (3% w/v carbopol in water), 3.5% w/w PG, 0.5% w/v cetomacrogol 1000, 12% w/w IPM, 4.5% w/w ST wax 30 and 4.5% w/v cetostearyl alcohol. A 10% w/v sodium hydroxide solution was used to neutralize the final formulation. All formulations were stored in glass borosilicate vials at 2–8°C and protected from light.

The BPQ suspension for oral dosing was prepared daily as 1 mg/mL in sodium acetate buffer (50 mM, pH 5). For the BPQ-HP-β-CD complex, a 16% HP-β-CD solution in sodium acetate (50 mM, pH 5) was prepared. The required amount of BPQ was added and the mixture constantly stirred for 72 h before filtering with a Durapor 0.45 μm filter.

The paromomycin–urea ointment was prepared as 15% paromomycin (as sulphate) and 10% urea formulated in a white soft paraffin base. 28

Statistical methods
Statistical analysis of the different topical formulations on both lesion size and parasite burden was performed using the Kruskal–Wallis [non-parametric analysis of variance (ANOVA)] and Dunnett’s multiple comparison tests (GraphPad InStat, version 3.05 for Windows 95/NT, GraphPad software, San Diego, CA, USA, www.graphpad.com). Statistical analysis of the VL treatment groups on parasite burden used a one-way ANOVA. The Kolmogorov–Smirnov test was used to ensure normal distribution of data. Results were considered significant when P < 0.05.

Histopathology
Whole skin lesions (one per treatment group) were fixed using formalin and sections were then stained using haematoxylin and eosin (H&E). Slides were examined by light microscopy, using ×1000 oil immersion. Photomicrographs were taken using a Zeiss Axioplan optical microscope and digital images captured with Optronics Magnafire LIVE.

Results
L. major-infected BALB/c (model for CL)
Comparison of treatment for early and late infection in CL. For the initial study, topical treatment was started 10 days post-infection and mice received a total of 14 daily applications for BPQ formulations. On the first day of treatment, lesions measured ~5 mm in diameter. Lesions treated with BPQ formulations were smaller and did not ulcerate in comparison with the untreated control. None of the lesions healed completely and therefore a mean lesion diameter was recorded (Figure 2).

Figure 2. Mean lesion diameter of BALB/c mice (infected with L. major JHSH118) treated with buparvaquone formulations versus control (n = 6 ± SEM).
BPQ-treated lesions had reduced mean lesion diameters. Two of the BPQ formulations, the o/w emulsion and hydrous gel, caused a significant decrease in the parasite burden, compared with the untreated control ($P < 0.0001$) (Figure 3).

In the second study, there was a delay in lesion appearance and treatment was only initiated 23 days post-infection. Mice received a total of 13 daily applications for BPQ and 3-POM-BPQ formulations. On the first day of treatment, lesions measured were \(~3\) mm in diameter. By the end of treatment, no treatment group showed a significantly different mean lesion size when compared with the untreated control ($P > 0.05$). However, two mice from the 3-POM-BPQ anhydrous gel group and one mouse from the 3-POM-BPQ hydrous gel group had re-epithelialized skin. All other mice had ulcerated lesions. Using a one-way ANOVA with Dunnett’s multiple comparison test (GraphPad InStat™), no formulation was found to be significantly different in parasite burden (real-time PCR), compared with the untreated control ($P > 0.05$).

In the third study, topical treatment was started 3 days post-infection to maximize the opportunity of demonstrating differences between the formulations. Mice received a total of 20 daily applications for BPQ and 3-POM-BPQ formulations. Lesions appeared \(~10\) days post-infection. Thirteen days after the start of treatment, all treated groups had significantly smaller lesions ($P < 0.0001$) than the untreated control (Figure 5). At 25 days post-infection, the untreated control mice had developed ulcerated lesions. Lesions had slowly begun to develop in a few of the treated groups, but were much smaller and none had ulcerated. Both the BPQ hydrous formulation and the 3-POM-BPQ anhydrous formulation significantly decreased parasite burden ($P < 0.001$) (Figure 6). Histopathology sections stained with H&E are shown in Figure 7. The untreated control section clearly shows a large infiltration of infected host cells throughout the dermal layer. Sections from treated mice did not show infected host cells or inflammatory response cells. This indicates that the topical treatment used in this study reduced parasite burden in the locally infected tissues. Although in this study, only one mouse was used per group for the histopathology samples.

Comparing the three studies, treatment of early stage infection showed the greatest differences between formulations. In particular, commencement of treatment prior to the appearance of any lesions produced a significant decrease in both the mean lesion diameter and parasite burden when compared with the untreated control.

**Differences in topical formulations.** In the initial study, all formulations were well tolerated. The percentage change in
the mean body weight of mice \((n = 5)\) includes untreated \(-0.4\%\), paromomycin–urea \(-0.8\%\), anhydrous gel \(+1.6\%\), hydrous gel \(+0.8\%\) and o/w emulsion \(-0.1\%\). Lesions treated with BPQ formulations were smaller (minimum 5.3 mm) and did not ulcerate in comparison with the untreated control (minimum 7.1 mm). Both the BPQ o/w emulsion and BPQ hydrous gel caused a significant decrease in parasite burden when compared with the untreated control \((P < 0.0001)\) (Figure 3). The group treated with the BPQ hydrous gel had less than half the parasite burden of the untreated control.

In the second study, all formulations were well tolerated, except for some slight erythema caused by the 3-POM-BPQ anhydrous gel. This formulation contained 30% v/v PG, which is known to cause irritation. The percentage change in the mean body weight of mice \((n = 5)\) includes untreated \(+8.7\%\), 3-POM-BPQ anhydrous gel \(+3.7\%\), hydrous BPQ w/o emulsion A \(+6.0\%\), BPQ w/o emulsion B \(+7.5\%\), BPQ hydrous gel \(+6.3\%\) and 3-POM-BPQ hydrous gel \(-0.2\%\). Two mice from the 3-POM-BPQ anhydrous gel group and one mouse from the 3-POM-BPQ hydrous gel group had re-epithelialized skin. However, there was no significant difference in either lesion size or parasite burden for any of the treatment groups in comparison with the untreated control. However, treatment with the BPQ hydrous gel did decrease the parasite burden.

In the third study, skin reactions were seen in several mice treated with anhydrous 3-POM-BPQ, although the skin condition improved by the study end. The erythema and oedema were graded as 3 and 2, respectively, by OECD guidelines. This was
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the only group that showed a decrease in the mean body weight and this is probably due to the relatively high concentrations of solvents used. Both ethanol and PG are known to cause irritation, and systemic uptake could also have occurred. One mouse in the BPQ emulsion group lost hair around the treated area and erythema/oedema occurred and the treatment was stopped after six doses. The skin condition in this mouse was graded as 2 for erythema by OECD guidelines. The percentage change in the mean body weight (n = 10) was untreated +4.0%, BPQ hydrous gel +2.4%, 3-POM-BPQ anhydrous gel −1.1%, BPQ w/o emulsion A +5.5% and BPQ in IPM +5.1%. Estimates of parasite burden at 22 days post-infection showed that both the BPQ hydrous gel and 3-POM-BPQ anhydrous gel caused a lower parasite burden in the mice than the untreated control (P < 0.001) (Figure 6). Similarly, the BPQ w/o emulsion had a significantly lower burden (P < 0.05). Estimates of parasite burden using the final titre method showed that all treatment groups had a reduced parasite burden by 26 days post-infection. In particular, the BPQ w/o emulsion showed a reduced parasite burden when compared with the untreated control at the three different time points when samples were taken (16, 22 and 26 days post-infection). For example, at 22 days post-infection, the group treated with the BPQ hydrous gel had less than one-fifth the parasite burden of the untreated control (Figure 6).

The formulations that showed the greatest efficacy (reduced lesion size and parasite burden) were the BPQ w/o emulsion, BPQ hydrous gel and the 3-POM-BPQ anhydrous gel. Both BPQ formulations were well tolerated. However, the 3-POM-BPQ anhydrous gel caused some local skin reactions.

**L. donovani-infected BALB/c (model for VL)**

The first VL experiment using *L. donovani*-infected BALB/c mice was carried out to investigate whether the BPQ prodrugs had improved activity when compared with BPQ (20 mg/kg for 5 days). Both the Pentostam control group and the prodrug groups had approximately two-thirds the parasite burden (shown by real-time PCR) of the untreated control (Figure 8). Using a Kruskal–Wallis (non-parametric ANOVA) test, the difference was significant (P < 0.0001). At the same molar equivalent, the BPQ-3-phosphate prodrug appeared to be the most effective. Estimates of parasite burden at 22 days post-infection showed that both the BPQ hydrous gel and 3-POM-BPQ anhydrous gel caused a lower parasite burden in the mice than the untreated control (P < 0.001) (Figure 6). Similarly, the BPQ w/o emulsion had a significantly lower burden (P < 0.05). Estimates of parasite burden using the final titre method showed that all treatment groups had a reduced parasite burden by 26 days post-infection. In particular, the BPQ w/o emulsion showed a reduced parasite burden when compared with the untreated control at the three different time points when samples were taken (16, 22 and 26 days post-infection). For example, at 22 days post-infection, the group treated with the BPQ hydrous gel had less than one-fifth the parasite burden of the untreated control (Figure 6).

The formulations that showed the greatest efficacy (reduced lesion size and parasite burden) were the BPQ w/o emulsion, BPQ hydrous gel and the 3-POM-BPQ anhydrous gel. Both BPQ formulations were well tolerated. However, the 3-POM-BPQ anhydrous gel caused some local skin reactions.

**Discussion**

BPQ was chosen for further studies as a topical formulation for CL and oral formulation for VL on the basis of its potent *in vitro* antileishmanial activity.13,15 It also has several physicochemical properties suitable for skin penetration (low molecular weight, low melting point, etc.). However, the low aqueous solubility of BPQ resulted in several phosphate prodrugs being investigated to improve aqueous solubility and enhance skin absorption.

The experiments on BALB/c mice infected with *L. major* indicate that topical formulations of both BPQ and its prodrug, 3-POM-BPQ, delay lesion progression and reduce parasite...
burden (up to seven times decrease). Several factors can cause differences in efficacy and are important in the evaluation of topicals. The timing for start of treatment can greatly influence the disease outcome. Topical treatment was started 3, 10 and 23 days post-infection in the three studies. Treatment started just 3 days post-infection had a more significant effect on lesion progression when compared with treatment started 10 or 23 days post-infection ($P < 0.001$). Initial screening of potential antileishmanials should be tested in early stage infections to maximize the opportunity for demonstrating differences in formulations. In this particular model, parasites have been shown to disseminate from the infection site within 24 h post-infection. The delay in treatment could have greatly affected the treatment outcome. In particular, an altered cytokine profile within the initial stages of infection is thought to influence disease progression. As the BALB/c L. major model is non-cure, the start of treatment early after infection is likely to provide the most sensitive model for drug screening. In future studies, a self-cure mouse model, such as C57Bl, could be investigated. Topical efficacy is likely to be more easily demonstrated in the early stages of infection than on well-established lesions. It has been well established in immunological studies that the course of infection can be manipulated with certain treatments (IL12, sublethal irradiation, anti-IL4 mAb, anti-CD4 mAb), but only in the early days after infection. Depending on a drug’s mode of action, this may also be the case for successful topical treatment. Evaluation of drug treatment for CL on established infections might miss potentially valuable formulations. In the clinical situation, patients usually only seek medical intervention on well-established lesions. However, simple human CL (L. major and L. mexicana) is a self-curing disease and the parasite burden is mainly confined to the site of infection. In comparison, the BALB/c mouse develops chronic progressive lesions and parasites are known to disseminate from the infection site within hours. In the second study, there was a pronounced delay in lesion appearance; the reason for this is not apparent. The infecting parasites were re-confirmed as being L. major and the calculations for infective dose inoculum were found to be correct. The effect of treatment should be investigated on both initial and late stage infections, as both the skin condition and parasite distribution can alter disease outcome.

Topical efficacy was determined by monitoring both lesion progression and parasite burden. The paromomycin ointment, which was initially included as a positive control, was shown to improve the clinical appearance of lesions; however, no significant decrease in parasite burden could be demonstrated. Therefore, evaluation of potential topical treatments for CL should include both parasite burden and lesion size for efficacy. Previous studies have usually not included both these criteria and their results have been difficult to interpret. For monitoring lesion progression, the mean lesion size was measured over the course of the study. Parasite burden was quantified by both LDAs and real-time PCR. Although both real-time PCR and the final titre method indicated significant decreases in parasite burden, the magnitude of decrease caused by the formulations varied between each of these methods. The real-time PCR method quantifies total kDNA, which can include both live and dead parasites. The LDAs are based on the ability of viable parasites to grow and replicate in culture. It is essential that suitable markers and less invasive markers are developed to enable measurement of drug activity (e.g. Katex). L. major parasite dissemination in BALB/c mice is known to occur within 10–24 h post-infection, so even a temporary reduction in parasite burden is likely to indicate successful local killing of parasites. Further studies would be interesting in a self-cure mouse strain (e.g. CBA) to determine whether treatment can prevent lesion appearance.

The differences in efficacy between the parent compound BPQ and the prodrug 3-POM-BPQ may be due to differences in formulation type, in addition to their intrinsic antileishmanial activity. Previous in vitro studies showed a different rank order of penetration for the formulations between the mouse and human skin. Therefore, formulations optimized both in vitro and in vivo on animal skin will probably behave quite differently when applied on human skin. Excipients can greatly influence skin penetration, so future studies should investigate optimizing the lead formulations and, in particular, determining stability for each one. It would also be worth testing further produgs of buparvaquone and optimizing each formulation for the particular skin type.

In general, the topical formulations were well tolerated, although some irritancy was noted with the 3-POM-BPQ anhydrous gel and BPQ w/o emulsion. The OECD test guideline 404 provides a grading scale for in vivo irritation, used by both US and UK regulatory authorities. Using these criteria, the 3-POM-BPQ anhydrous gel caused erythema and oedema in several mice between grade 1 and 2. These might be due to the prodrug itself or the released BPQ. Improvements in bioavailability can increase toxicity as well as efficacy. One excipient in this formulation is propylene glycol, which was previously shown to cause no signs of irritancy after 24 h contact in hairless mice at 100%. PG is GRAS listed in the FDA inactive ingredients and is classed as a minimal irritant. The main excipient, ethanol, is a known mild irritant (especially >50%) and may cause contact dermatitis. Further development of formulations should aim to reduce irritancy. The bad skin reaction (grade 3 erythema/oedema), which occurred due to the BPQ w/o emulsion, was only seen in one mouse. Owing to its severity, dosing was stopped in this particular mouse and the skin recovered over the following days.

In the L. donovani studies, both BPQ produgs caused a significant decrease in the liver parasite burden. Both studies indicated that the prodrug, buparvaquone-3-phosphate, was the most effective at reducing parasite burden. The difference between produgs could be due to several factors, for example, stability, rate of parent compound release or pharmacokinetics. In the second study, BPQ was administered orally as a solution in the solvents IPM and PEG400. These were chosen because of good BPQ solubility and oral excipient tolerability. The availability of the drug in solution as opposed to suspension is likely to improve dissolution within the gastrointestinal tract and ultimately enhance bioavailability. These results confirm improved efficacy with the BPQ produgs and this is most likely due to enhanced absorption and greater bioavailability. The increased solubility in the solvents also led to a much greater reduction in parasite burden when compared with the suspension formulation of BPQ at the equivalent dose. This highlights the importance of solubility in absorption and also choosing the optimal excipients as a drug vehicle for in vivo evaluations. The improved efficacy of the produgs also confirms that optimizing physicochemical properties can greatly enhance bioavailability and efficacy. These results confirm that buparvaquone is a potent antileishmanial and warrants further investigation of both BPQ and its phosphate produgs for the treatment of VL.
Buparvaquone as an antileishmanial

Apart from the development of paromomycin topicals (in the 1980s), there have been few advances in the treatment of CL. Excipients should also be chosen that are suitable not only for the drug but also for the end user. The use of methylbenzethonium chloride in one of the paromomycin ointments highlights this fact, as it is known to cause skin irritancy. The introduction of a topical formulation, such as buparvaquone, would be a significant advance for the treatment of simple CL. In particular, the avoidance of the parenteral antimonials would greatly increase patient compliance and reduce treatment costs.

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Transparency declarations

None to declare.

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