Journal of Antimicrobial Chemotherapy Advance Access published August 21, 2007

Journal of Antimicrobial Chemotherapy
doi:10.1093/jac/dkm303

JAC

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

Tracy Garnier[1]*†, Antti Mäntylä[2], Tomi Järvinen[2], Jayne Lawrence[3], Marc Brown[3]† and Simon Croft[1,4]

[1]Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK; [2]Department of Pharmaceutical Chemistry, University of Kuopio, PO Box 1627, FI-70211 Kuopio, Finland; [3]Pharmaceutical Sciences Research Division, Kings College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK; [4]Drugs for Neglected Diseases Initiative (DNDi), 1 Place St Gervais, Ch-1201 Geneva, Switzerland

Received 8 February 2007; returned 24 April 2007; revised 25 May 2007; accepted 13 July 2007

***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

*Correspondence address. School of Pharmacy, University of Hertfordshire, College Lane, Hatfield AL10 9AB, UK.
Tel: +44-1707-284392; Fax: +44-1701-284506; E-mail: t.garnier@herts.ac.uk
†Present address. School of Pharmacy, University of Hertfordshire, College Lane, Hatfield AL10 9AB, UK.

associated with many problems (i.e. resistance, toxicity and cost).[3] Recent advances have included the introduction of miltefosine and newer formulations of existing drugs, for example, liposomal amphotericin B.[4,5]

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.[6] 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).[7] Studies in the Old World show intralesional administration to give superior healing rates when compared with intramuscular antimonials.[8] 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. However, these paromomycin ointments possess problems of varying efficacy and toxicity.[9–12]

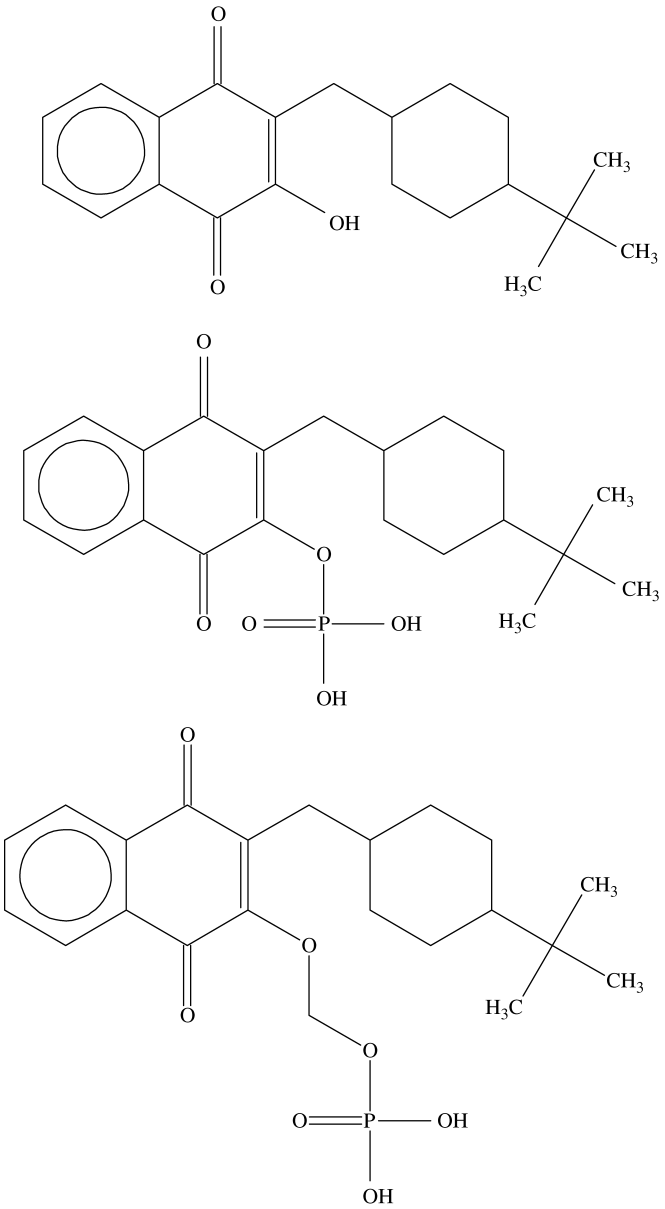

Figure 1. Structure of buparvaquone (326.43 Da), buparvaquone-3-phosphate (406.42 Da) and 3-POM-buparvaquone (436.45 Da); shown from top to bottom.

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.*,[13] 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-phosphonooxymethyl-buparvaquone (3-POM-BPQ), were shown to have potent *in vitro* antileishmanial activity (nanomolar range) against species causing both visceral and cutaneous leishmaniasis.[14] Formulations of BPQ and 3-POM-BPQ were subsequently shown to penetrate both human epidermal and full thickness BALB/c mouse skin *in vitro*.[15]

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

Properties	BPQ	BPQ-3-phos	3-POM-BPQ
Log *D*	7.02 (pH 3.0)	1.87 (pH 3.0), 1.27 (pH 5.0), 0.47 (pH 7.4)	2.96 (pH 3.0), 1.83 (pH 5.0), 1.16 (pH 7.4)
pK_a	5.70	2.68; 6.74	2.28; 6.76
Melting point (°C)	ND	121.9–124.0	ND
Aqueous solubility (mg/mL)	<0.03 μg/mL (pH 3.0, pH 5.0); 0.03 μg/mL (pH 7.4)	>3.5 (pH 3.0, pH 5.0, pH 7.4)	>3.5 (pH 3.0, pH 5.0, pH 7.4)

ND, not determined.
Adapted from Mäntylä *et al.*[14]

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 (Coventry, UK). Isopropyl myristate (IPM), polyethylene glycols (PEG300/400), mineral oil, glycerin, paromomycin (as sulphate) and hydroxypropl-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 *ad libitum*. 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 stabilates 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* HU3 inoculated intravenously (maximum 0.2 mL) in the lateral tail vein with 1.5×10^7 freshly harvested amastigotes. After infection, mice were marked for individual identification and randomly allocated into groups of five. After 7 days, a mouse was sacrificed and parasitic burden was based on microscopic enumerations of amastigotes against host cell nuclei on liver impression smears.[18]

Dosing started on the seventh day post-infection for 5 continuous days. As a positive control, one group received 15 mg Sb^v/kg (as sodium stibogluconate) subcutaneously for 5 days, as described previously.[19,20] In this study, an ED_{50} was determined to be 13.2 ± 3.75 mg Sb^v/kg. The oral formulations were made 24 h prior to dosing each day (except the BPQ-HP-β-CD complex). These included BPQ, the BPQ-HP-β-CD complex, 3-POM-BPQ and BPQ-3-phosphate in 50 mM sodium acetate buffer, pH 5. The first experiment used an oral dose of 20 mg/kg BPQ (molar equivalent for prodrugs) for 5 days and the second study used 50 mg/kg BPQ (molar equivalent for prodrugs) for 5 days. Animals were 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 10^6–10^7 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.[7,23] Cutaneous lesions were measured weekly using digital callipers (Jencons^{TM}) 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 o/w emulsion (0.89% w/w). Mice were infected with $\sim 2.2 \times 10^6$ *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.

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 $\sim 2.0 \times 10^6$ *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 $\sim 2.0 \times 10^6$ *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

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 69 504). 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™ SYBRGreen 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 χ^2 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/w 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/w carbopol ETD2020 mucilage (3% w/v carbopol in water), 3.5% w/w PG, 0.5% w/w cetomacrogol 1000, 12% w/w IPM, 4.5% w/w ST wax 30 and 4.5% w/w 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) and shaken vigorously just prior to administration using a maximum volume of 0.4 mL (20 mg/kg and then 50 mg/kg). Both BPQ-3-phosphate and 3-POM-BPQ were dosed at the same molar equivalent to BPQ for each study. Suspensions were prepared daily 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™ SP 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).

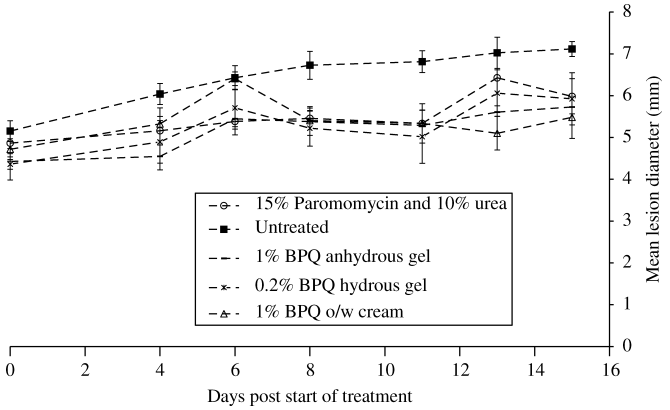

Figure 2. Mean lesion diameter of BALB/c mice (infected with *L. major* JISH118) treated with buparvaquone formulations versus control ($n = 6 \pm$ SEM).

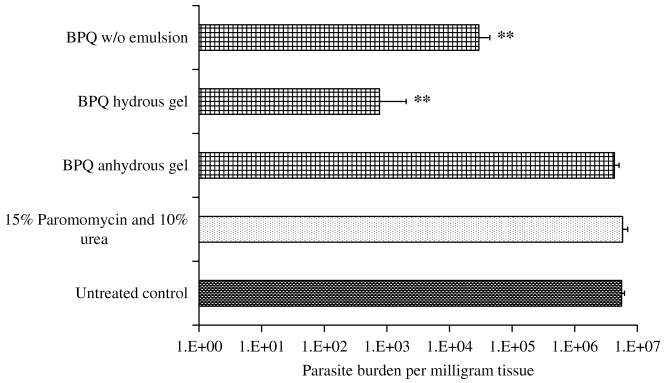

Figure 3. Skin parasite burden per milligram tissue sample ($n = 3 \pm$ SEM) for BALB/c mice (infected with *L. major* JISH118) treated with buparvaquone formulations as determined by real-time PCR.

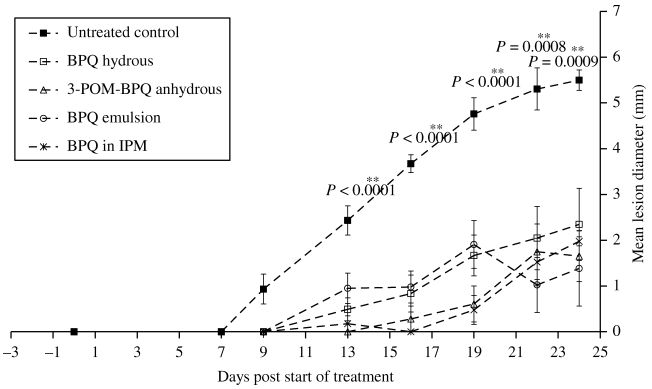

Figure 5. Mean lesion diameter versus time of BALB/c mice (infected with *L. major* JISH118) treated with BPQ and 3-POM-BPQ formulations (P values from one-way ANOVA using Dunnett's multiple comparison post-test), $n = 10 \pm$ SEM.

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 (Figure 4, $P > 0.05$). 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

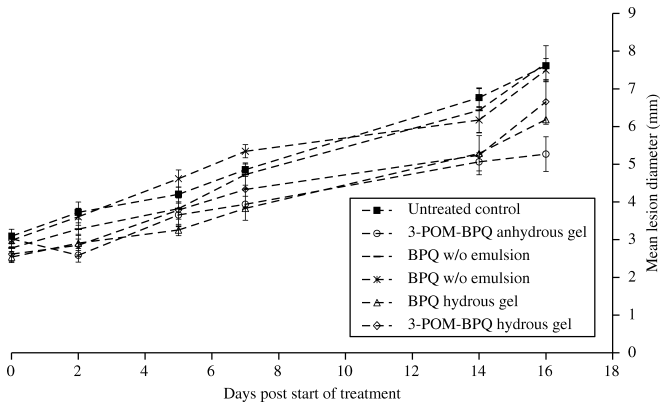

Figure 4. Mean lesion diameter of BALB/c mice (infected with *L. major* JISH118) treated with BPQ versus 3-POM-BPQ formulations ($n = 6 \pm$ SEM).

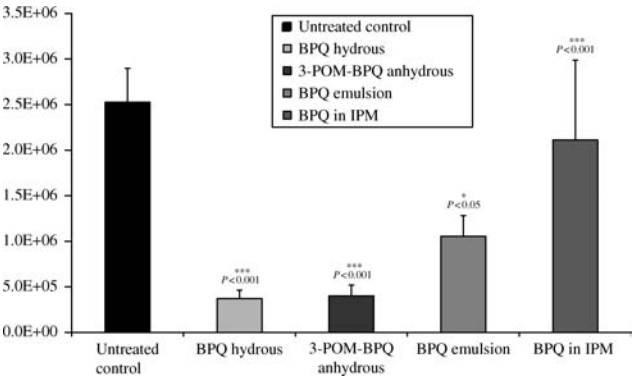

Figure 6. Parasite burden per milligram tissue sample for BALB/c mice (infected with *L. major* JISH118) treated ($n = 3 \pm$ SEM) 22 days post-infection, as determined by real-time PCR.

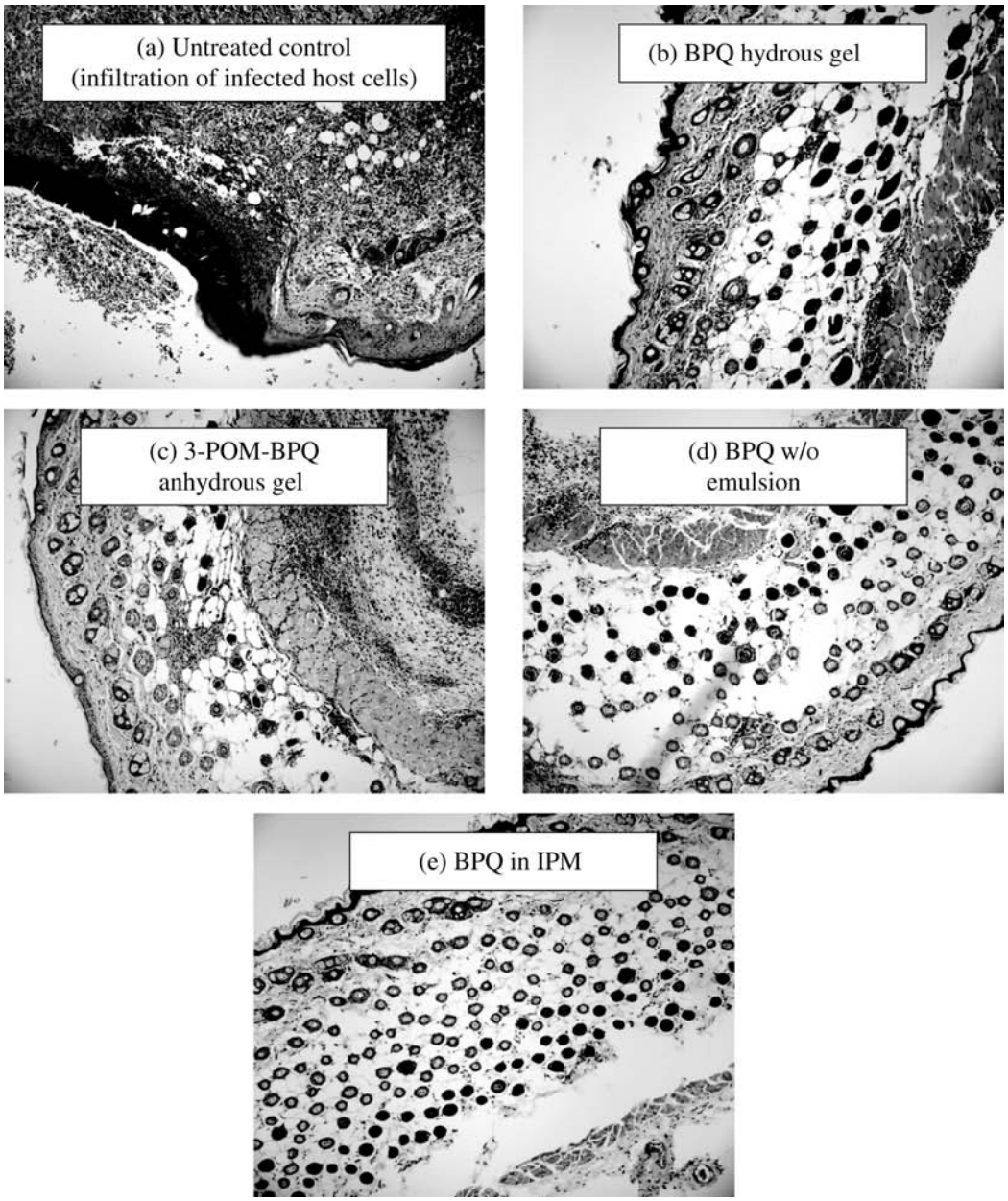

Figure 7. Histopathology sections for BALB/c mice (infected with *L. major* JISH118) treated with: (a) untreated control; (b) BPQ hydrous gel; (c) 3-POM-BPQ anhydrous gel; (d) BPQ w/o emulsion and (e) BPQ in IPM (H&E stain, magnification $\times 100$).

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

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.

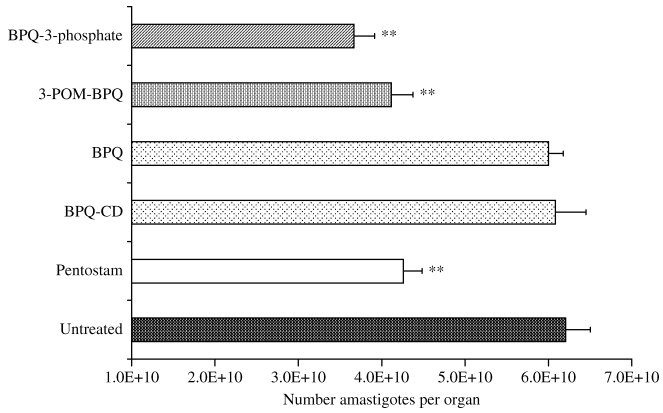

Figure 8. Liver parasite burden for BALB/c mice infected with *L. donovani* HU3 ($n = 5 \pm$ SEM) dosed at 20 mg/kg BPQ (molar equivalent for prodrugs) for 5 days, as determined by real-time PCR.

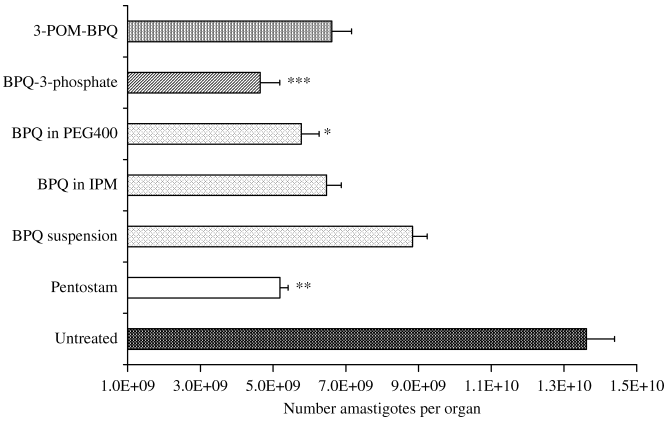

Figure 9. Liver parasite burden for BALB/c mice infected with *L. donovani* HU3 ($n = 5 \pm$ SEM) dosed at 50 mg/kg BPQ (molar equivalent for prodrugs) for 5 days, as determined by real-time PCR.

Neither the BPQ suspension nor BPQ-CD formulation caused a significant decrease in parasite burden. No toxic effects were noted in any of the groups, although the BPQ-CD group was the only one to have a reduced mean body weight post-treatment (∼1.6% decrease).

The second experiment investigated higher doses using 50 mg/kg for 5 days. The effect of increasing BPQ solubility was also examined by using solvents, which had animal toxicity data to support oral dosing.[16] Liver smears again indicated that both BPQ prodrugs significantly reduced parasite burden (Figure 9). Using a Kruskal–Wallis test (non-parametric ANOVA), the difference was significant ($P = 0.0003$). The BPQ-3-phosphate prodrug was again shown to be the most effective drug at reducing parasite numbers (Figure 9). The parasite burden in the BPQ-3-phosphate group was ∼34% of the parasite burden in the untreated control. Both BPQ treatment groups, IPM and PEG400, reduced the parasite burden to a greater extent than the BPQ suspension (50 mM sodium acetate buffer pH 5). A higher BPQ dose was used than in the last study and the BPQ suspension did reduce parasite burden but was least effective of all the formulations. However, it should be noted that the parasite burden for the untreated control was greater in the first *L. donovani* study than in the second study. The liver parasite burden for the untreated control in the first *in vivo* was approximately four times higher. Toxic effects were seen in the 3-POM-BPQ group, which included a reduction in the mean body weight, poor hair condition and a hunched appearance.

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.[29] 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.[21,30,31] 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.[29] 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[13,15] 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 prodrugs 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.[32] 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%.[33] 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 prodrugs 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 prodrugs 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 prodrugs 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 prodrugs 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 prodrugs for the treatment of VL.

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.

Acknowledgements

We wish to thank Mr Lesley Etheridge (London School of Hygiene and Tropical Medicine) for his help with the sectioning and histopathology and Dr Pascale Kropf (Imperial College, London) for her help with the LDAs.

Funding

This work was financially supported by the Sir Halley Stewart Trust.

Transparency declarations

None to declare.

References

1. Desjeux P. Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 2004; **27**: 305–18.

2. Murray HW. Clinical and experimental advances in treatment of visceral leishmaniasis. *Antimicrob Agents Chemother* 2001; **45**: 2185–97.

3. Croft SL, Coombs GH. Leishmaniasis—current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol* 2003; **19**: 502–8.

4. Berman JD. Recent developments in leishmaniasis: epidemiology, diagnosis, and treatment. *Curr Infect Dis Rep* 2005; **7**: 33–8.

5. Meyerhoff A. U.S. Food and Drug Administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. *Clin Infect Dis* 1999; **28**: 42–51.

6. Murray HW, Berman JD, Davies CR *et al*. Advances in leishmaniasis. *Lancet* 2005; **366**: 1561–77.

7. Garnier T, Croft SL. Topical treatment for cutaneous leishmaniasis. *Curr Opin Investig Drugs* 2002; **3**: 538–44.

8. Alkhawajah AM, Larbi E, al-Gindan Y *et al*. Treatment of cutaneous leishmaniasis with antimony: intramuscular versus intralesional administration. *Ann Trop Med Parasitol* 1997; **91**: 899–905.

9. El-On J, Livshin R, Even-Paz Z *et al*. Topical treatment of cutaneous leishmaniasis. *J Invest Dermatol* 1986; **87**: 284–8.

10. Soto J, Hernandez N, Mejia H *et al*. Successful treatment of New World cutaneous leishmaniasis with a combination of topical paromomycin/methylbenzethonium chloride and injectable meglumine antimonate. *Clin Infect Dis* 1995; **20**: 47–5.

11. Ben Salah A, Zakraoui H, Zaatour A *et al*. A randomized, placebo-controlled trial in Tunisia treating cutaneous leishmaniasis with paromomycin ointment. *Am J Trop Med Hyg* 1995; **53**: 162–6.

12. Asilian A, Jalayer T, Whitworth JA *et al*. A randomized, placebo-controlled trial of a two-week regimen of aminosidine (paromomycin) ointment for treatment of cutaneous leishmaniasis in Iran. *Am J Trop Med Hyg* 1995; **53**: 648–51.

13. Croft SL, Hogg J, Gutteridge WE *et al*. The activity of hydroxynaphthoquinones against *Leishmania donovani*. *J Antimicrob Chemother* 1992; **30**: 827–32.

14. Mäntylä A, Garnier T, Rautio J *et al*. Synthesis, *in vitro* evaluation, and antileishmanial activity of water-soluble prodrugs of buparvaquone. *J Med Chem* 2004; **47**: 188–95.

15. Garnier T, Mäntylä A, Järvinen T *et al*. Topical buparvaquone formulations for the treatment of cutaneous leishmaniasis. *J Pharm Pharmacol* 2007; **51**: 41–9.

16. Wade A, Weller PJ. *Handbook of Pharmaceutical Excipients*. London: Pharmaceutical Society of Great Britain, 1994

17. Yardley V, Croft SL. Animal models of cutaneous leishmaniasis. In: Sande M, Zak O, eds. *Handbook of Animal Models of Infection*. London: Academic Press, 1999; 775–81.

18. Stauber LA, Franchino EM, Grun J. An eight-day method for screening compounds against *Leishmania donovani* in golden hamster. *J Protozool* 1958; **5**: 269–73.

19. Neal RA. Experimental chemotherapy. In: Peters W, Killick-Kendrick R, eds. *The Leishmaniases in Biology and Medicine*. London: Academic Press, 1987; 794–845.

20. Yardley V, Croft SL. A comparison of the activities of three amphotericin B lipid formulations against experimental visceral and cutaneous leishmaniasis. *Int J Antimicrob Agents* 2000; **13**: 243–8.

21. Kropf P, Etges R, Schopf L *et al*. Characterisation of T cell-mediated responses in nonhealing and healing *Leishmania major* infections in the absence of endogenous IL-4. *J Immunol* 1997; **159**: 3434–43.

22. da Silva R, Sacks DL. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Infect Immun* 1987; **55**: 2802–6.

23. Neal RA, Murphy AG, Olliaro P *et al*. Aminosidine ointments for the treatment of experimental cutaneous leishmaniasis. *Trans R Soc Trop Med Hyg* 1994; **88**: 223–5.

24. Nicholas L, Prina E, Lang T *et al*. Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues. *J Clin Microbiol* 2002; **40**: 1666–9.

25. Kropf P, Muller I, Brunson K. The leishmaniasis model. In: Kaufmann SHE, Kabelitz D, eds. *Immunology of Infection. Series: Methods in Microbiology, 32*. London: Academic Press, 2002; 419–58.

26. Titus RG, Marchand M, Boon T *et al*. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. *Parasite Immunol* 1985; **7**: 545–55.

27. Taswell C. Limiting dilution assays for the separation, characterization and quantitation of biologically active particles and their clonal progeny. In: Pretlow TG, Pretlow TP, eds. *Cell Separation: Methods and Selected Applications*. London: Academic Press, 1987; 109–45.

28. El-On J, Jacobs GP. Compositions and methods for topical treatment of cutaneous leishmaniasis with paromomycin. US patent 4,505,901, 1985.

29. Laskay T, Diefenbach A, Rollinghoff M *et al*. Early parasite containment is decisive for resistance to *Leishmania major* infection. *Eur J Immunol* 1995; **25**: 2220–7.

30. Bretscher PA, Wei G, Menon JN *et al*. Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to *Leishmania major*. *Science* 1992; **257**: 539–42.

31. Rogers KA, DeKrey GK, Mbow ML *et al*. Type 1 and type 2 responses to *Leishmania major*. *FEMS Microbiol Lett* 2002; **209**: 1–7.

32. Bagley DM, Gardner JR, Holland G *et al*. Skin irritation: reference chemicals data bank. *Toxicol In Vitro* 1996; **10**: 1–6.

33. Philips CA, Michniak BB. Topical application of Azone analogs to hairless mouse skin: a histopathological study. *Int J Pharm* 1995; **125**: 63–71.