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Topical buparvaquone formulations for the treatment of cutaneous leishmaniasis

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Abstract

As the part of a study to develop buparvaquone (BPQ) formulations for the treatment of cutaneous leishmaniasis, the topical delivery of BPQ and one of its prodrugs from a range of formulations was evaluated. In previous studies, BPQ and its prodrugs were shown to be potent antileishmanials in-vitro, with ED50 values in the nanomolar range. 3-Phosphono-oxymethyl-buparvaquone (3-POM-BPQ) was the most potent antileishmanial and was chosen, together with the parent drug, for further investigation. The ability of the parent and prodrug formulations to cross human and murine skin was tested in-vitro using the Franz diffusion cells. Formulations intended for topical application containing either BPQ or 3-POM-BPQ were developed using excipients that were either acceptable for topical use (GRAS or FDA inactive ingredients) or currently going through the regulatory process. BPQ was shown to penetrate both human epidermal membranes and full thickness BALB/c skin from a range of formulations (gels, emulsions). Similarly, 3-POM-BPQ penetrated full-thickness BALB/c skin from several gel formulations. In-vitro binding studies showed that BPQ bound melanin in a dose-dependent manner and preferably bound to delipidized skin over untreated BALB/c skin (on a weight to weight basis). The results confirm that BPQ and its prodrug 3-POM-BPQ can penetrate the skin from several formulations, making them potentially interesting candidates for further investigation of topical formulations using in-vivo models of cutaneous leishmaniasis.

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Introduction

Leishmaniasis is a worldwide disease caused by protozoan parasites of the genus *Leishmania*. *Leishmania* species cause a range of diseases in man, ranging from disfiguring cutaneous leishmaniasis (CL) to visceral leishmaniasis, which 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) (Desjeux 2004). Treatment mainly relies on the parenteral administration of pentavalent antimonials (e.g. intramuscular or intravenous sodium stibogluconate 20 mg kg^{-1} per day for 20 days), which are associated with many problems (resistance, toxicity, cost) (Croft & Coombs 2003). Recent advances in the treatment of leishmaniasis have included the introduction of miltefosine (Berman 2005) and newer formulations of existing drugs, for example liposomal amphotericin B (Meyerhoff 1999). However, for self-limiting forms of CL (such as *Leishmania major* and *Leishmania mexicana*), topical therapy probably offers the most acceptable form of treatment. In CL, the disease is normally localized to the site of infection within dermal macrophages. Local treatment remains an attractive approach for the simple localized forms of CL that are not at risk of more complex manifestations. For more serious presentations of the disease, involving vital organs or mucosal membranes, more aggressive systemic therapy is usually warranted. Local therapy approaches have included physical methods (cryotherapy, thermotherapy, surgical removal and electrotherapy), paromomycin ointment, intralesional antimony and ethanolic amphotericin B solutions. CL causes ulcerative lesions that are often disfiguring and can leave permanent scars. Typically, papules develop at the site of infection, enlarge to a nodule and progress to ulcerated lesions that last less than a year (Murray et al 2005). Multiple lesions and disfiguring scars can create a life-long stigma. Treatment aims to accelerate healing, minimize

scarring and prevent the development of more complex manifestations such as mucocutaneous leishmaniasis and diffuse CL. Advantages of topical therapy include reduced cost (hospitalization not required), lower toxicity (drug targeted to infected tissues) and patient compliance (ease of administration) (Garnier & Croft 2002). Currently, there are only two topical formulations commercially available for the treatment of CL: both ointment formulations contain the aminoglycoside, paromomycin. However, these paromomycin ointments exhibit varying efficacy and have toxicity problems (El-On et al 1986; Asilian et al 1995; Ben Salah et al 1995; Soto et al 1995).

Buparvaquone (BPQ) is a hydroxynaphthoquinone, structurally related to the antiprotozoan drugs, parvaquone and atovaquone. In previous studies, BPQ was shown to exhibit antileishmanial efficacy both in-vitro and in-vivo against visceral leishmaniasis models. In *Leishmania donovani*-infected BALB/c mice, BPQ was administered subcutaneously in corn oil (100 mg kg^{-1} per day for 5 days) and produced a 62% suppression of hepatic amastigote burden (Croft et al 1992). This study also reported an ED50 of 0.08 μM for *L. donovani* promastigotes after 24 h incubation. Later studies found BPQ to be active against both promastigotes and amastigotes of a number of *Leishmania* species at concentrations in the low nanomolar range (Mäntylä et al 2004). However, disappointing results were obtained after treating dogs infected with visceral leishmaniasis with intramuscular BPQ (4×5 mg kg^{-1}), as treatment failed to stop the progress of the disease (Vexenat et al 1998). To date, there have been no published studies on the efficacy of BPQ against CL.

Ideally, a topical drug candidate should have optimal physicochemical properties for percutaneous absorption. These include a low molecular weight (<500 Da), low melting point, log $P_{octanol}$ 1–3, solubility parameter 9–10, and have few functional groups capable of hydrogen bonding (Hadgraft & Pugh 1998). Often drug properties are not ideal and pharmaceutical formulations are therefore designed to optimize skin absorption and drug targeting. BPQ is a lipophilic molecule (log $D_{pH3.0}$ 7.02) (Mäntylä et al 2004). Highly lipophilic molecules (log D > 3) tend to remain within the stratum corneum and do not penetrate into the lower more hydrophilic dermal layer of the skin. Issues of poor aqueous solubility focused the aim of BPQ topical development on enhancing drug loading and increasing skin penetration. Less lipophilic phosphate prodrugs were designed with the aim of altering physicochemical properties and improving BPQ skin permeation (Mäntylä et al 2004). Two BPQ prodrugs have been synthesized, namely buparvaquone-3-phosphate and 3-phosphonooxymethyl-buparvaquone (3-POM-BPQ). 3-POM-BPQ was chosen in the present study for development as a topical formulation since it had a better partitioning profile (log $D_{pH5.0}$ 1.83) and flux rate in human skin (1.5 ± 0.12 nmol cm^{-2} h^{-1} at a pH of 5.0) compared with buparvaquone-3-phosphate (Mäntylä et al 2004).

The aim of this study was to identify, evaluate and characterize formulations of BPQ using in-vitro human and murine skin models, since the mouse is used for the in-vivo model of CL (Yardley & Croft 2000), as part of a project to develop a new treatment for CL.

Materials and Methods

Materials

BPQ was from GlaxoWellcome, Beckenham, UK (Figure 1A). The BPQ prodrugs, 3-POM-BPQ (Figure 1B) and

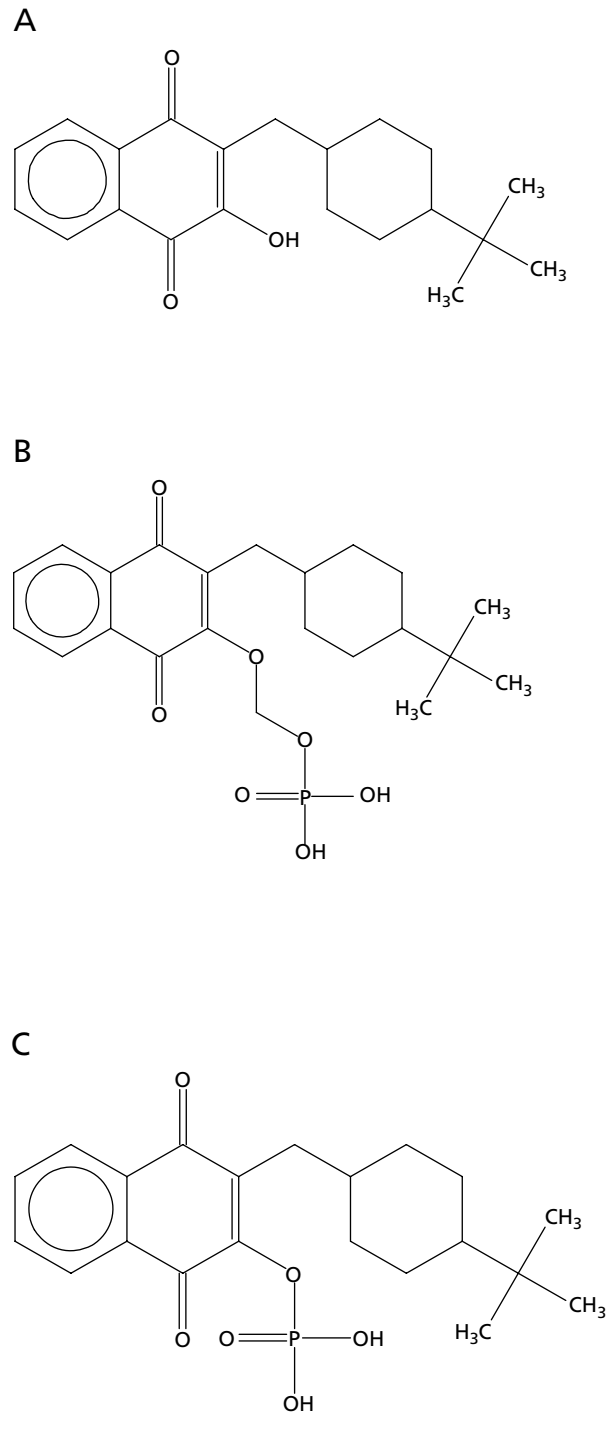

Figure 1 A. Structure of buparvaquone (326.43 Da). B. Structure of 3-phosphono-oxymethyl-buparvaquone (436.45 Da). C. Structure of buparvaquone-3-phosphate (406.42 Da).

buparvaquone-3-phosphate (Figure 1C), were synthesized at Kuopio University, Finland (Mäntylä et al 2004). All chemicals and solvents were of analytical grade (AnalaR, ACS). The formulations used only FDA approved or GRAS listed excipients (Kibbe 2000; http://www.fda.gov/), with the exception of the novel silicones from Dow Corning (Coventry, UK), which are currently undergoing regulatory evaluation (Kibbe 2000; http://www.dowcorning.com). The Franz diffusion cells, purchased from Soham Scientific (Soham, UK), had a mean radius and mean volume of receptor fluid of 0.48 ± 0.02 cm and 2.52 ± 0.09 cm^3, respectively.

The following excipients were obtained from Sigma (Poole, UK): hydroxypropyl-β-cyclodextrin (HP-β-CyD), mineral oil, propylene glycol (PG), polyethylene glycol 300 (PEG300), polyethylene glycol 400 (PEG400), isopropyl myristate (IPM), glycerin and melanin powder. Ethanol, sodium hydroxide and sodium chloride were obtained from BDH (Poole, UK). Cetostearyl alcohol was obtained from Paroxite Ltd (London, UK). Cyclomethicone 5NF, dimethiconol blend 20, silky wax 10 and emulsifier 10 were obtained from Dow Corning (Seneffe, Belgium). The water used was deionized from Millipore Q, except in the case of HPLC grade water which was supplied by Fischer (Loughborough, UK). Cetomacrogol 1000 was obtained from Rhodia (Watford, UK). Klucel HF Pharm (hydroxypropyl cellulose) was obtained from Hercules (Hopewell, VA, USA). Carbopol ETD 2020 (acrylates/C10–30 alkyl acrylate crosspolymer) was obtained from Noveon (Cleveland, OH, USA).

HPLC analysis

The HPLC assay for BPQ was based on a previously published method (Kinabo & Bogdan 1988). In brief, a Waters HPLC system with UV detector set at 252 nm was used. The isocratic method used a silica-C18 (5 μm particle size) reverse-phase column (LISPRP8E-5-125AF) and a pre-column guard (LISPRP8E-5-10C). The mobile phase consisted of a mixture of 0.05 M sodium acetate (pH 3.6) and methanol. The mobile phase for BPQ and the pro-drug 3-POM-BPQ contained 15% and 22% v/v of the aqueous phase, respectively. The flow rate was 1.3 mL min^{-1} and the injection volume 20 μL. The retention time for BPQ was 5 min. The pro-drug method had a retention time of 3 min for 3-POM-BPQ and 10 min for BPQ.

Skin sources

Human skin was obtained with informed consent and King's College London Research Ethics Committee approval, from female patients who had undergone elective abdominal plastic surgery. Heat-separated epidermal membranes were obtained by immersing full-thickness skin in water at 60°C for 45 s (Kligman & Christophers 1963) and were mounted on Whatman filter paper (no. 1). Full-thickness murine skin (shaven) was excised from dead female BALB/c mice (Harlan Sera-Lab, Loughborough, UK), from lumbar regions. Fatty deposits and connective tissue were carefully removed using a fine-tipped forceps. Discs of human and mouse skin were cut from the excised skin on a Teflon dissecting board using a sharpened cork borer (13.5 mm diameter). Skin samples were stored at −20°C and used within 3 months.

Formulation development

Initially the solubility of both BPQ and 3-POM-BPQ was investigated in a range of solvents. BPQ and 3-POM-BPQ were formulated as a saturated solution in a range of solvents (namely ethanol, HP-β-CyD, IPM, PEG400 and PG), since this allowed in-vitro comparison under steady-state conditions. Excipients were chosen because of solvent capabilities (increases drug loading), penetration enhancement (increases skin penetration) and their regulatory status. A saturated receiver solution was included for BPQ (2% w/v HP-β-CyD in PBS, pH 7.4). Silicones were chosen as topical excipients since they have been shown to enhance wound healing (Sweitzer 2006) and they have physicochemical properties ideal for topical formulations (non-greasy, low surface tension, good aesthetics) (Séné et al 2002). Silicones have a long history of medicinal use and are widely used as a material for medical devices (catheters and pacemakers) and implants.

Anhydrous BPQ gels consisted of approximately 80% w/w ST elastomer 10, 19% w/w cyclomethicone 5NF and 1% w/w IPM. Anhydrous gels containing 3-POM-BPQ were prepared using approximately 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 was used as a gelling agent (<2% w/w). The hydrous gels consisted of approximately 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 gel formulations.

The BPQ water-in-oil (w/o) emulsion (A) consisted of approximately 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 approximately 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 oil-in-water (o/w) emulsion consisted of approximately 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.

In-vitro diffusion studies

Release and permeation studies, using Franz diffusion cells, were carried out using a previously published method (Howes et al 1996). The receiver fluids for BPQ and 3-POM-BPQ were 2–5% HP-β-CyD in PBS (pH 7.4) and 2–5% HP-β-CyD in acetate buffer (pH 5), respectively. Both fluids had been validated to ensure sink conditions were maintained over the time course of the study (Mäntylä et al 2004). Steady-state flux (J_{ss}; infinite dose) or penetration rate (finite dose) was determined from the slope of the straight portion of the line, plotting cumulative amount per unit area against time. Extrapolation of this line to the intercept was defined as the lag time.

Drug release rates from both BPQ (hydrous gel, w/o emulsion (A), w/o emulsion (B), anhydrous gel) and 3-POM-BPQ formulations (anhydrous gel (A), anhydrous gel (B), hydrous gel) were measured through a semi-permeable membrane of regenerated ethyl cellulose (SpectraPor 4 REC membrane (MWCO 12 000–14 000), 132 498, lot 28870) using a similar method to that of Arnardottir et al (1996). Formulations (100 μL) were applied to the surface of the membrane and receiver fluid samples were assayed for drug by HPLC over 280 min.

The first skin study investigated the penetration of BPQ from a range of saturated solvent solutions (ethanol, IPM, PEG 400 and PG) across human epidermal membranes (54-year old female, white, European). Samples (500 μL) of each saturated solution were applied to the surface of the skin and the receiver fluid assayed for BPQ by HPLC over 24 h. The permeability coefficient (k_p) of BPQ was calculated by dividing J_{ss} by the concentration of BPQ in each formulation. The second skin study investigated the penetration of BPQ from the saturated solutions across full-thickness BALB/c skin. Saturated BPQ solutions (200 μL) were applied and receiver fluid assayed for BPQ by HPLC over 48 h.

Penetration of 3-POM-BPQ across full-thickness BALB/c skin from saturated solutions was investigated. A 500-μL dose was applied to the surface of the skin and both the drug remaining on the surface of the skin and that in the upper layers of the skin (depth profiling) carried out 26 h after application (Surber et al 1999). In order to recover drug remaining on the surface (unabsorbed drug), the skin surface was swabbed with two cotton buds (previously soaked in methanol). To validate the recovery method, a known amount of formulation was placed on the skin surface and then immediately removed by the swabbing protocol and the amount of drug recovered checked against the amount applied. Depth profiling was achieved by tape-stripping the stratum corneum using Scotch tape (3M, Bracknell, UK). The first strip was taken as unabsorbed drug on the skin surface. Stripping of the skin was continued until the membrane became too fragile and tore (Qureshi et al 1998; Bashir et al 2001; Couteau et al 2001). Drug was extracted from each piece of tape using methanol and assayed using HPLC.

BPQ penetration from several emulsions (o/w and w/o) and gels (anhydrous and hydrous) across full-thickness BALB/c skin was investigated. A 10-μL dose of the various formulations was applied to the surface of the skin. Recovery from the surface of the skin was determined 8 h after application. A final study compared the penetration of BPQ and 3-POM-BPQ from a 10-μL application of a range of formulations across full-thickness BALB/c skin. Surface recovery was determined at 8 h.

Binding studies

The methods for determining drug binding to both melanin and skin components have been previously described (Banning & Heard 2002; Heard et al 2003). Briefly, for the binding to melanin study, 400 μL of a range of BPQ solutions (10 mM, 5 mM, 1 mM, 700 μM, 200 μM and 100 μM in a 1:1 v/v mixture of PEG 400:PBS, pH 7.4) were added to 2.5 mg of melanin powder and then incubated on an autostirrer (500 rev min^{-1}) at 37°C for 24 h. The resultant suspensions were placed in Millipore Ultrafree Eppendorf tubes (Sigma) and centrifuged at 4779 *g* for 10 min (Heraeus Biofuge Pico) to separate free from bound drug. Bound drug was extracted from recovered melanin by sonicating for 10 min with mobile phase (a mixture of 0.05 M sodium acetate, pH 3.6; containing 15% and 22% v/v of the aqueous phase for BPQ and 3-POM-BPQ, respectively, and methanol) used for HPLC analysis and the resulting extract assayed by HPLC.

Briefly, to investigate the binding of BPQ to skin components, BALB/c skin discs (full-thickness skin) were delipidized as previously described (Wertz & Downing 1987). Weighed skin samples were placed in glass scintillation vials together with 1 mL of drug solution (BPQ in IPM at 0.1, 0.5, 1, 2 and 5 mg mL^{-1}) and left to incubate at 37°C for 24 h. The epidermal samples were then transferred to Millipore Ultrafree Eppendorf tubes (Sigma) and centrifuged at 4779 *g* for 30 min. Skin-bound drug was recovered by extracting three times using a total of 1 mL mobile phase. The three extracts were pooled and the pooled samples were assayed by HPLC. The results were graphed as mg (mg tissue)$^{-1}$ versus the initial incubation concentration.

Statistical methods

Statistical analysis of the different formulations on the amount of drug penetration or percentage recovery at each time point was performed using a one-way analysis of variance. The one-way analysis of variance with Tukey Kramer multiple comparison tests were performed using GraphPad InStat version 3.05 for Windows 95/NT (GraphPad Software, San Diego, CA, USA).

For the binding studies, statistical analysis of the amount of bound BPQ was used to compare the delipidized skin to normal skin, using a paired two-tailed *t*-test (GraphPad InStat). The amount of melanin-bound drug was used to statistically analyse and compare BPQ with another antileishmanial, sitamaquine (Garnier et al 2006), using a paired two-tailed *t*-test (GraphPad InStat).

Results

In-vitro diffusion studies

Release studies

Drug release rates across the synthetic membrane (penetration profiles not shown) were similar for the BPQ formulations, with the w/o emulsion (A) having the greatest rate at approximately 0.22 mg cm^{-2} min^{-1} and the BPQ hydrous gel having the lowest rate at approximately 0.17 mg cm^{-2} min^{-1}. There was no significant difference in release rates between the four BPQ formulations ($P > 0.05$, Tukey Kramer multiple comparisons). The release rate was greatest for 3-POM-BPQ from the anhydrous gels (approx. 40 mg cm^{-2} min^{-1}). Both anhydrous gels released a significantly greater amount of 3-POM-BPQ at all time points compared with the hydrous gel ($P < 0.05$, Tukey Kramer multiple comparisons). The 3-POM-BPQ hydrous gel only had a release rate of approximately 10 mg cm^{-2} min^{-1}, although the hydrous gel concentration (0.32% w/w) was much lower than the anhydrous formulations

(2.30% and 3.12% w/w). This experiment confirmed both BPQ and 3-POM-BPQ were released from the various formulations tested.

Saturated solutions

Initial studies determined the rate of BPQ penetration across human epidermal membranes from a range of saturated solvents (Table 1). The general rank order for the penetration rate of BPQ from the various solvents was ethanol > HP-β-CyD solution > PG > IPM > PEG 400, with a J_{ss} of $1.64 \pm 0.41\ \mu g\ cm^{-2}\ h^{-1}$ when ethanol was used and a J_{ss} of $0.47 \pm 0.02\ \mu g\ cm^{-2}\ h^{-1}$ when PEG 400 was used as a solvent. The highest k_p value of 1.74 ± 0.14 was obtained when PG was used as solvent.

By way of contrast, the study determining the penetration rate of BPQ across full-thickness BALB/c skin, again using saturated solvents, indicated that the general rank order was IPM > ethanol > HP-β-CyD solution > PEG400 > PG. IPM gave the greatest J_{ss} rate of $3.61 \pm 0.20\ \mu g\ cm^{-2}\ h^{-1}$ (Table 1). In this experiment, BPQ in ethanol gave the highest k_p value of 1.31 ± 0.10. At the 48-h time-point, the cumulative amount of BPQ which had crossed into the receiver fluid from IPM, was significantly greater from that seen with the other solvents ($P < 0.001$, one-way analysis of variance). BPQ penetration from ethanol was also significantly greater compared with HP-β-CyD, PEG400 and PG ($P < 0.01$, one-way analysis of variance). These experiments confirmed that BPQ penetrated both human epidermal skin and full-thickness BALB/c from a range of solvents, although BPQ more readily penetrated the latter skin type.

The J_{ss} rates for 3-POM-BPQ across full-thickness BALB/c skin from various saturated solvents are shown in Table 2. For comparison, the values obtained for BPQ performed at the same time are shown. 3-POM-BPQ showed the greatest permeation through BALB/c skin from saturated solutions of IPM and ethanol, exhibiting a J_{ss} of 242.54 ± 13.97 and $247.69 \pm 60.42\ \mu g\ cm^{-2}\ h^{-1}$, respectively. The cumulative amounts of 3-POM-BPQ and BPQ penetrating receiver fluid from IPM and ethanol were significantly greater than when either PG or PEG400 were used as solvent ($P < 0.001$, one-way analysis of variance). The calculated lag times for 3-POM-BPQ in IPM and ethanol were 3.4 and 3.1 h, respectively. Tape-stripping results indicated greatest skin retention from 3-POM-BPQ in ethanol (Figure 2). The amounts recovered from tape strips decreased progressively with increasing

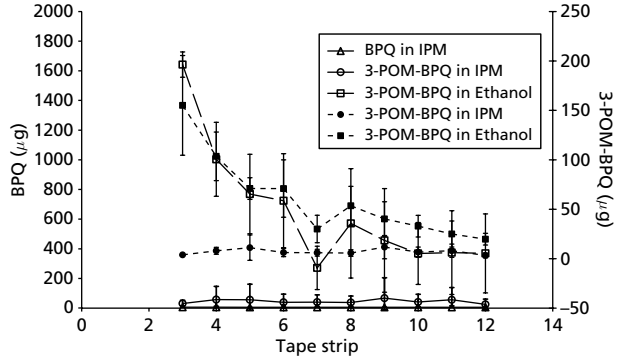

Figure 2 Depth profile for buparvaquone (BPQ) and 3-phosphono-oxymethyl-buparvaquone (3-POM-BPQ) in mouse skin (mean ± s.e.m., n = 3). IPM, isopropyl myristate.

Table 1 Steady state flux rate (J_{ss}) and permeability coefficient (k_p) for buparvaquone solvent systems (mean ± s.e.m., n = 6).

Formulation	Human epidermal skin		Full-thickness BALB/c skin	
	J_{ss} ($\mu g\ cm^{-2}\ h^{-1}$)	k_p ($\times 10^{-3}$ cm h^{-1})	J_{ss} ($\mu g\ cm^{-2}\ h^{-1}$)	k_p ($\times 10^{-3}$ cm h^{-1})
Ethanol	1.64 ± 0.41	0.63 ± 0.16	2.89 ± 0.22	1.31 ± 0.10
IPM	0.49 ± 0.04	0.13 ± 0.01	3.61 ± 0.20	0.48 ± 0.03
2% HP-β-CyD in PBS	0.72 ± 0.24	0.69 ± 0.23	1.17 ± 0.24	1.23 ± 0.25
PEG400	0.47 ± 0.02	0.13 ± 0.01	0.80 ± 0.13	0.15 ± 0.02
PG	0.56 ± 0.05	1.74 ± 0.14	0.62 ± 0.11	0.97 ± 0.17

IPM, isopropyl myristate; HP-β-CyD, hydroxypropyl-β-cyclodextrin; PBS, phosphate buffered saline; PEG400, polyethylene glycol 400; PG, propylene glycol.

Table 2 3-Phosphono-oxymethyl-buparvaquone (3-POM-BPQ) solvent systems for the BALB/c skin study (mean ± s.e.m., n = 6).

Sample	Assay of samples by HPLC			
	% w/w BPQ	% w/w 3-POM-BPQ	Total BPQ (%)[a]	BPQ flux rate ($\mu g\ cm^{-2}\ h^{-1}$)
BPQ in IPM	0.71	—	0.71	8.89 ± 0.64
3-POM-BPQ in IPM	4.00	0.97	4.72	242.54 ± 13.97
3-POM-BPQ in ethanol	11.88	8.70	18.39	247.69 ± 60.42
3-POM-BPQ in PG	2.35	0.82	2.96	8.80 ± 1.11
3-POM-BPQ in PEG400	9.65	5.52	13.78	4.41 ± 0.99

[a]Assumes ~74.79% buparvaquone content in 3-POM-BPQ. IPM, isopropyl myristate; PG, propylene glycol; PEG400, polyethylene glycol 400;

skin depth, suggesting that most of the drug resided in the upper layers of the skin.

Formulations

The BPQ penetration rate across full-thickness BALB/c skin was greatest from the IPM solution ($2.36 \pm 0.40\ \mu g\ cm^{-2}\ h^{-1}$) and the least penetration was from the o/w emulsion ($0.16 \pm 0.02\ \mu g\ cm^{-2}\ h^{-1}$) (penetration profiles not shown). The cumulative amount of BPQ obtained when IPM was used as a solvent was significantly greater than that obtained using any of the other formulations ($P < 0.001$, one-way analysis of variance). The overall rank order of BPQ penetration was greatest for the IPM solution > hydrous gel > w/o emulsion (A) > anhydrous gel > o/w emulsion. The greatest percent penetration at 8 h was seen when the anhydrous gel was used, while the least percent penetration was seen when the o/w emulsion (Table 3). Regardless of the formulation, between 67–87% of the applied dose was not recovered, which may be a consequence of either skin retention and/or inadequate recovery technique. However, this study did confirm that BPQ could penetrate full-thickness BALB/c skin when formulated in a range of formulations, all of which contained IPM.

The penetration rate of both BPQ and 3-POM-BPQ incorporated in a range of formulations was then determined across BALB/c skin (penetration profiles not shown). The greatest rate of flux was achieved when 3-POM-BPQ was formulated in an anhydrous gel B ($1.63 \pm 0.13\ \mu g\ BPQ\ cm^{-2}\ h^{-1}$; $0.40 \pm 0.20\ \mu g$ 3-POM-BPQ $cm^{-2}\ h^{-1}$) (Table 4). In comparison, the greatest rate of flux of BPQ was seen when the hydrous gel was used, with a value of $0.47 \pm 0.03\ \mu g\ cm^{-2}\ h^{-1}$.

Table 3 Mass balance recovery study for buparvaquone formulations after 8 h dose exposure (mean ± s.e.m., n = 6).

Formulation	% Penetrated by 8 h	% Recovery from wash	% Unrecovered
IPM	7.64 ± 1.10	4.58 ± 0.22	87.78 ± 0.90
Hydrous gel	11.79 ± 1.16	6.80 ± 0.14	81.41 ± 1.10
w/o emulsion (A)	11.19 ± 1.55	6.65 ± 0.22	82.16 ± 1.39
Anhydrous gel	22.60 ± 0.78	10.39 ± 0.13	62.07 ± 0.84
o/w emulsion	2.81 ± 0.23	10.74 ± 0.52	86.45 ± 0.49

IPM, isopropyl myristate.

Table 4 Buparvaquone (BPQ) and 3-phosphono-oxymethyl-buparvaquone (3-POM-BPQ) formulations for the BALB/c skin study (mean ± s.e.m., n = 6).

Formulation	Concentration BPQ % w/w	BPQ penetration rate [3-POM-BPQ] ($\mu g\ cm^{-2}\ h^{-1}$)
BPQ hydrous gel	0.08	0.47 ± 0.03
BPQ w/o emulsion (A)	0.25	0.43 ± 0.03
3-POM-BPQ anhydrous (gel A)	2.30	1.22 ± 0.11 [0.45 ± 0.02]
3-POM-BPQ anhydrous (gel B)	3.11	1.63 ± 0.13 [0.40 ± 0.02]
3-POM-BPQ hydrous gel	0.32	1.16 ± 0.06 [0.39 ± 0.02]

Overall, the formulations containing 3-POM-BPQ resulted in significantly greater amounts of BPQ penetrating the receiver fluid than either of the BPQ formulations tested ($P < 0.001$, one-way analysis of variance). Indeed there was no significant difference between the amounts of 3-POM-BPQ penetrating BALB/c skin from any of the formulations tested. However, as the aqueous stability of 3-POM-BPQ is known to be relatively low (Mäntylä et al 2004), any prodrug present in the receiver fluid will eventually be hydrolysed to release BPQ. The percentage of BPQ unrecovered (Figure 3), was greatest from the w/o emulsion (A) containing BPQ and the hydrous gel formulation of 3-POM-BPQ. The percentage of unrecovered 3-POM-BPQ was also greatest from the hydrous gel. The percentage of BPQ unrecovered from the w/o emulsion (A) was significantly greater than any of the other formulations, while the amount of BPQ recovered form the anhydrous 3-POM-BPQ formulation was significantly lower ($P < 0.001$, one-way analysis of variance).

Binding studies

The binding of BPQ to melanin appeared to be linear and dose dependent (Figure 4). The melanin binding of BPQ to

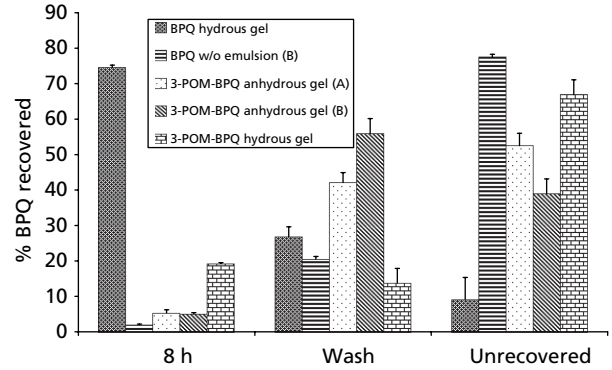

Figure 3 Percentage recovery of buparvaquone (BPQ) from applied dose (mean ± s.e.m., n = 6).

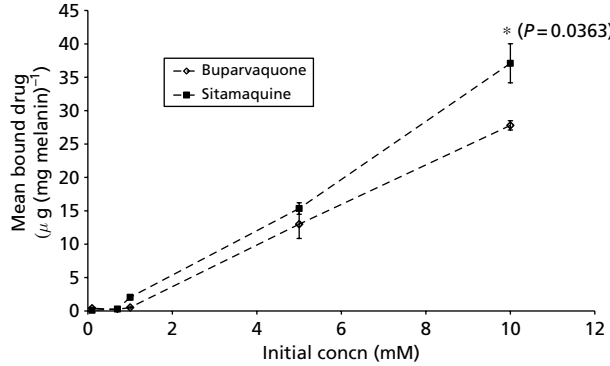

Figure 4 Binding of buparvaquone to melanin (*Sepia officinalis*) as a function of the initial amount incubated (mean ± s.e.m., n = 3). *$P < 0.05$, two-tailed *t*-test. Sitamaquine dihydrochloride is shown for comparison.

melanin was compared with that seen for another antileishmanial, sitamaquine dihydrochloride, which has recently been evaluated as a topical candidate for CL (Garnier et al 2006). At the highest incubation concentration, BPQ was significantly less bound to melanin compared with sitamaquine ($P = 0.0363$, two way t-test). This is perhaps not unexpected because sitamaquine is more hydrophilic than BPQ, which exhibits limited solubility in aqueous environments. Finally, the binding of BPQ to untreated mouse skin and delipidized mouse skin showed that BPQ bound to delipidized mouse skin to a significantly greater extent than the untreated skin ($P < 0.05$, two-way t-test) (Figure 5). In both cases, the binding against concentration profiles were linear, suggesting that the binding sites were not saturated, under the conditions studied.

Discussion

BPQ and 3-POM-BPQ have been shown to be potent antileishmanials, with nanomolar ED50 values in in-vitro models of *L. donovani* promastigotes and amastigotes (Croft et al 1992; Mäntylä et al 2004). Of the two pro-drugs, 3-POM-BPQ had the greatest intrinsic anti-leishmanial activity and was therefore chosen for further investigation.

Release studies confirmed both BPQ and 3-POM-BPQ were released from the various formulations tested. Preliminary in-vitro flux data indicated that BPQ flux across human epidermal layers and full-thickness BALB/c skin was greatest from a saturated solution of ethanol and IPM, respectively. Both IPM and ethanol are known to enhance skin penetration via the intercellular lipid pathway (Walker & Smith 1996). IPM is known to interact with stratum corneum lipids and extract cholesterol to cause structural deformations within the lipid bilayers (Arellano et al 1999) and may therefore be expected to promote intercellular penetration of a lipophilic molecule such as BPQ. Ethanol, in contrast, enhances skin penetration by several mechanisms, including increasing drug solubility in the vehicle, enhancing drug partitioning into the stratum corneum, lipid extraction or by increasing drug thermodynamic activity in the vehicle during evaporation (Williams & Barry 2004). A high k_p for BPQ indicates a poor affinity for the vehicle (Table 1). Therefore, both PG and ethanol would be expected to favour partitioning into human and BALB/c skin, respectively.

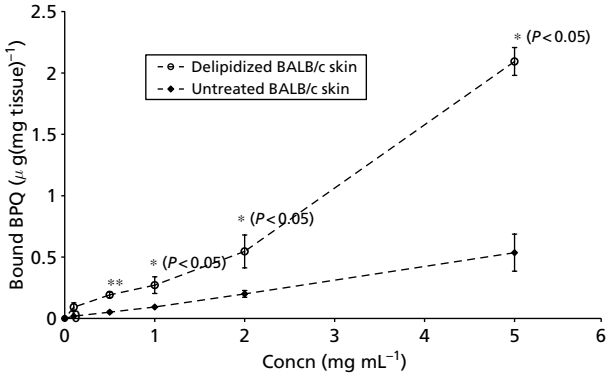

Figure 5 Buparvaquone (BPQ) binding to full-thickness BALB/c skin (native and delipidized) (mean ± s.e.m., n = 4). *$P < 0.05$, two-tailed t-test.

Not surprisingly, the results indicated that BPQ more readily penetrated full-thickness BALB/c skin than human epidermal skin. The difference in general rank order of skin penetration from the various saturated solutions across human epidermal and BALB/c skin is owing to variations in skin composition and structure. Such differences are not uncommon. For example, a study comparing extracted lipids and stratum corneum thickness found 60.5 μg extracted lipid cm^{-2} and a thickness of 18.2 μm for human skin, and 212.4 μg extracted lipid cm^{-2} and a thickness of 8.8 μm for hairless mouse skin. Also murine skin lacks sweat glands but has more follicles than human skin (Magnusson et al 2001). Beckley-Kartey et al (1997) determined the thickness of mouse stratum corneum to be 6 μm, with a hair follicle density of ~650 cm^{-2}, compared with 17 μm and 10 cm^{-2}, respectively, for human skin. The results for human and BALB/c skin in the present study are not directly comparable as the human epidermal layer did not contain the dermis. This is a significant difference because for a very lipophilic compound such as BPQ, diffusion through the hydrophilic domain (viable epidermis, dermis) can be rate limiting. The presence of the dermis in full-thickness BALB/c skin may alter the overall rates of skin penetration.

Since BPQ exhibits a good solubility in IPM (>4 mg mL^{-1}) and readily penetrated BALB/c skin, several formulations containing IPM were developed. Formulation studies in BALB/c skin demonstrated a general rank order of BPQ penetration as follows: hydrous gel > w/o emulsion > anhydrous gel > o/w emulsion. Since BPQ is a highly lipophilic molecule, it was thought that a hydrous formulation would provide the greatest release rate owing to greater thermodynamic activity within this environment. Other factors such as ionization of the drug (which depends on formulation pH and drug pK_a), skin hydration and presence of excipients may also play a role. Comparing the two emulsions, the greater penetration from the w/o emulsion is probably due to BPQ being predominantly present in the external oleaginous phase. The o/w emulsion on the other hand would mainly consist of BPQ in the internal oily phase and would be expected to provide a more sustained release action. Future studies should focus on developing topical BPQ formulations such as the hydrous gel and the w/o emulsion (A). In addition, the development of a transdermal patch would be worth investigating for visceral leismaniasis.

Ethanol was chosen as the solvent for the 3-POM-BPQ containing formulations since it provided good solubility (>350 μg mL^{-1}) and stability of the drug. The BPQ hydrous gel and w/o emulsion (A) were then chosen for comparison with the prodrug 3-POM-BPQ formulations. The first study using solvent systems showed 3-POM-BPQ to have the greatest J_{ss} from IPM. Tape-stripping data, however, indicated greatest retention in the upper skin surface to be achieved from ethanol. However, as topically applied excipients may alter keratinocyte cohesion (Surber et al 1999), tape stripping may remove different amounts of skin due to variations in cell adhesion. Therefore, this method can only give an indication of skin uptake. The greatest 3-POM-BPQ penetration

rate was from the anhydrous gel formulation. Overall, in comparison with the BPQ-containing formulations, the formulations containing 3-POM-BPQ provided much greater skin penetration. Future studies should focus on developing 3-POM-BPQ formulations such as the anhydrous gel A.

Having demonstrated in in-vitro experiments that BPQ was being retained in BALB/c skin, binding studies were undertaken to investigate its possible interactions with various skin components. In-vitro binding studies showed BPQ bound melanin in a dose-dependent manner, although to a lesser extent when compared with the hydrophilic molecule, sitamaquine. The quinone structure present in BPQ is known to have an affinity for melanin and many drugs have previously been shown to bind melanin (antimalarial, fluoroquinolones, chlorpromazine) (Larsson 1993). For example, the quinone lawsone (2-hydroxy-1,4-naphthoquinone) is the main component of the natural dye henna and this is well known to accumulate within melanin rich tissue such as skin and hair (Nohynek et al 2004). The melanin used in this study was derived from *Sepia officinalis* (common or European cuttlefish) and although differences are likely between species and tissues, it provides a good indication of the likely binding profile in human skin. Melanin is a polyanion that contains several carboxyl and semiquinone groups (Fukuda et al 2000) and binding may involve H-bonding between the melanin hydroxyl and oxygen groups of BPQ. Depending on the interaction, binding may provide a drug reservoir that is slowly released over time or, on the other hand, it may lead to inactivation or subtherapeutic levels at the site of action.

BPQ was also shown to bind more preferably to delipidized skin over untreated BALB/c skin. Although the delipidization method does not remove covalently bound lipid, delipidized skin is essentially proteinaceous in nature. The greater binding of BPQ to delipidized skin suggests that BPQ has an affinity for the keratinocyte proteins. BPQ is a lipophilic molecule and hydrophobic interactions might result in unspecific binding to proteins. These results suggest that BPQ interacts with protein components within the BALB/c skin and one mechanism for penetration therefore may involve the transcellular route. However, the extremely low aqueous solubility of BPQ would imply that it predominantly penetrates BALB/c skin via the lipophilic intercellular pathway. Most drugs are believed to penetrate skin by both the intercellular and transcellular routes (Hadgraft 2001). These results suggest that BPQ is also likely to interact with protein components within the skin. Further studies would be required to confirm the predominant route of skin absorption.

Conclusions

Previous results from in-vitro parasite models confirmed the high potent antileishmanial activity (nanomolar range) of BPQ and pro-drugs against *L. donovani* against a range of *Leishmania* species that cause cutaneous disease providing the basis for further studies.

Formulations were developed according to the different physicochemical properties of both BPQ and 3-POM-BPQ. BPQ was shown to penetrate both human and mouse skin in-vitro. Both the BPQ hydrous gel (containing IPM, carbopol ETD 2020 and water) and BPQ w/o emulsion (A) (containing mineral oil, IPM, emulsifier 10, NaCl and water) had the greatest penetration rates across full-thickness BALB/c skin. The prodrug 3-POM-BPQ was shown to readily penetrate full-thickness BALB/c skin from an anhydrous gel A (containing PEG300, ethanol, PG). In-vitro skin studies showed that BALB/c skin was more readily permeable to compounds compared with human epidermal membranes. Also, comparative studies with several formulations showed a different rank order of penetration between the mouse and human skin. Therefore, it is important to understand how formulations optimized both in-vitro and in-vivo on animal skin will behave when applied to human skin. These results warrant further investigation into the topical formulations of BPQ for CL. Since the development of paromomycin topicals in the 1980s (El-On et al 1984), there have been few advances in the topical treatment of CL. Recently, a Phase II placebo-controlled trial reported efficacy for a topical formulation of aminoglycosides (WR279396) against Old World CL (Ben Salah et al 2005). Excipients should also be chosen that are suitable not only for the drug but also for the end user. The use of methylbenzathonium chloride in one of the paromomycin ointments highlights this fact (El-On et al 1986), as it is known to cause skin irritancy. The introduction of a topical formulation, such as BPQ, 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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