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In-vitro and in-vivo studies on a topical formulation of sitamaquine dihydrochloride for cutaneous leishmaniasis

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Abstract

The efficacy of topical formulations of the 8-aminoquinoline, sitamaquine dihydrochloride, in both in-vitro and in in-vivo models of cutaneous leishmaniasis is reported. In-vitro parasite assays confirmed that sitamaquine dihydrochloride was active against a range of *Leishmania* species that cause either cutaneous or visceral leishmaniasis, with ED50 values against amastigotes over the range of 2.9 to 19.0 μM. A range of topical sitamaquine dihydrochloride formulations (anhydrous gel, emulsions) were developed for studies on experimental cutaneous leishmaniasis using only topically acceptable excipients or those currently undergoing regulatory approval. An uptake study into murine skin confirmed in-vitro skin penetration and retention. Several formulations were tested in-vivo against *Leishmania major* cutaneous lesions in BALB/c mice. None of the sitamaquine dihydrochloride formulations tested appeared to either slow lesion progression or reduce parasite burden.

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Introduction

Leishmaniasis is a widespread disease caused by protozoan parasites of the genus *Leishmania* that cause a range of diseases in man, ranging from disfiguring cutaneous leishmaniasis (CL) to visceral leishmaniasis (VL), which is fatal if left untreated. CL is the most common form of leishmaniasis and has an annual incidence of 1 to 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 (i.e. resistance, toxicity, cost) (Croft & Coombs 2003). Recent advances 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*), local therapy offers a more 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, not at risk of more complex manifestations. Dissemination may occur when certain species escape to mucosal tissue (i.e. mucocutaneous leishmaniasis) or multiple cutaneous sites. 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. Studies in the Old World show that intralesional administration gives superior healing rates compared with intramuscular antimonials (Sharquie et al 1988; Alkhawajah et al 1997; Mujtaba & Khalid 1999). Advantages of this route include targeting higher drug concentrations to the site of infection, lower systemic toxicity, decreased cost and faster healing time. 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, which last less than a year (Murray et al 2005). Multiple lesions and disfiguring scars can create a lifelong stigma. Treatment aims to accelerate healing, minimize scarring and prevent the development of more complex manifestations such as mucocutaneous leishmaniasis and diffuse

cutaneous leishmaniasis. Advantages of topical therapy include reduced cost (avoid hospitalization), lower toxicity (target drug to infected tissues) and increased patient compliance (non-invasive administration) (Garnier & Croft 2002). 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 (El-On et al 1992; Ben Salah et al 1995; Soto et al 1995; Asilian et al 2003).

Sitamaquine is an 8-aminoquinoline, previously shown to be active against experimental models of VL (Kinnamon et al 1978; Neal et al 1985), as well as the fungus *Pneumocystis carinii* (Bartlett et al 1991; Queener et al 1992) and the protozoa *Trypanosoma cruzi* (Chiari et al 1996) and *Babesia microti* (Marley et al 1997). Currently, sitamaquine is in Phase II clinical trials for the oral treatment of VL (Sherwood et al 1994; Dietze et al 2001; Jha et al 2005). Although the in-vitro activity of sitamaquine against *Leishmania* species that cause CL has been reported (Berman & Lee 1983; Callahan et al 1997), activity was poor in mouse models of CL (Bjorvatn & Neva 1979; Peters et al 1980; Neal et al 1985). There have been no previous published studies on the topical delivery of sitamaquine.

Ideally, a topical drug candidate should have optimal physicochemical properties for percutaneous absorption. These are a small 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. Sitamaquine dihydrochloride has a molecular weight of 416.43 Da, m.p. 179°C and is known to be soluble in both aqueous and non-aqueous solvents. It has 1 hydrogen bond donating and 3 hydrogen bond accepting groups. Sitamaquine dihydrochloride was chosen for development as a topical formulation for CL since it had physicochemical properties suitable for skin absorption and it was known to have antileishmanial activity.

In the present study, the activity of sitamaquine dihydrochloride against several species of *Leishmania* that cause CL was first determined in-vitro to confirm intrinsic drug sensitivity, a variable factor between species for other drugs (Croft et al 2002). Pre-formulation and skin permeation studies were then carried out to optimize sitamaquine dihydrochloride formulations, which were then investigated for efficacy using the animal model for CL. The formulations were developed for local delivery of sitamaquine dihydrochloride to infected tissues rather than transdermal delivery into the systemic circulation. The parasites causing CL are known to reside within dermal macrophages.

Materials and Methods

Materials

Sitamaquine salt (6-methoxy-8(6-diethylaminohexylamino)lepidine dihydrochloride) was a gift from GlaxoSmithKline, UK. Paromomycin (as sulfate) was obtained from Sigma, Poole, UK. All chemicals and solvents were of the highest grade available. Benzene ring labelled, ^{14}C sitamaquine dihydrochloride, with a specific activity of 58 mCi mmol^{-1} (radioactive concentration 137.3 μCi mg^{-1}) was donated by GlaxoSmithKline, UK (manufactured by Amersham, UK). The formulations were prepared using only FDA approved or GRAS listed excipients (Kibbe 2000; http://www.fda.gov/), with the exception of the novel silicones, which were supplied by Dow Corning (Coventry, UK). The Franz diffusion cells (Soham Scientific, Soham, UK) had a mean radius and mean volume ($\pm$ s.d.) of 0.48 ± 0.02 cm and 2.52 ± 0.09 cm^3, respectively. The following excipients were obtained from Sigma, UK: mineral oil, propylene glycol (PG), polyethylene glycol 300 (PEG300), polyethylene glycol 400 (PEG400), isopropyl myristate (IPM), propylene carbonate (PC), glycerine, sodium dodecyl sulfate (SDS) and melanin powder. White soft paraffin was obtained as Vaseline. Poloxamer F127 was obtained from BASF Corp, Berlin, Germany. Ethanol 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. Both Soluene-350 and Hionic-Fluor were obtained from Packard Instruments B.V., Chemical Operations, Groningen, Holland. The water used was deionized from Millipore Q, except in the case of HPLC grade water which was supplied from Fischer, Loughborough, UK.

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 mounting on Whatman filter paper (No. 1). Full-thickness murine skin (shaven) was excised from lumbar regions of dead female BALB/c mice (Harlan Sera-Lab, Loughborough, UK). Fatty deposits and connective tissue was carefully removed using a fine-tipped forceps. Discs for both 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.

Animals

Female BALB/c mice were obtained from Harlan Sera-Lab, UK, and weighed approximately 20 g each at the time of infection. A standard rodent diet (SDS R and M No. 1 expanded) and deionized water were supplied *ad libitum*. Female golden hamsters (*Mesocricetus auratus*), used for the routine passage of *Leishmania donovani*, and outbred CD1 mice, used to isolate peritoneal macrophages (Mϕs), 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 70/04 779).

Parasites

Promastigotes of *L. major* (MHOM/SA/85/JISH118); *Leishmania panamensis* (MHOM/PA/67/BOYNTON); *L. mexicana* (MHOM/BZ/82/BEL21); *L. mexicana* (MNYC/BZ/62/M379); *Leishmania aethiopica* (Khartoum); *Leishmania amazonensis* (MORY/BR/72/M1824) and *L. donovani* (MHOM/ET/67/HU3) were taken from liquid nitrogen stabilates and cultured in Schneider's *Drosophila* medium (GibcoBRL, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (HI-FCS) (Harlan Sera-Lab, UK). They were maintained at 26°C. *L. donovani* amastigotes were isolated from the female golden hamster spleen (*M. auratus*).

In-vitro *Leishmania* models

Drug activity against the extracellular promastigote and intracellular amastigote was determined using the Alamar Blue assay and peritoneal macrophage (Mϕ) assay, respectively, as previously described in Mäntylä et al (2004). Briefly, the promastigotes or infected Mϕs were incubated for 72 h in the presence of either media alone or serial drug dilutions. Results for both assays are reported as ED50 values with 95% confidence intervals, calculated using Microsoft Xlfit (version 2.0.2). Data were fitted using the non-linear sigmoidal curve-fitting Levenburg Marquardt algorithm.

Formulations

The paromomycin/urea ointment was used as a standard in in-vivo studies (Neal et al 1994). This consisted of 15% w/w paromomycin (as sulfate) and 10% w/w urea ointment, formulated in the oleaginous base, white soft paraffin.

Sitamaquine dihydrochloride was initially formulated as a gel (either anhydrous or hydrous) for preliminary in-vitro permeation studies. The gels were prepared by first making 5 mL of the solution according to the formulae listed in Table 1. These solutions were saturated with sitamaquine dihydrochloride. After saturation, Carbomer 934 (Carbopol 934) was added as a gelling agent (final concentration $< 1\%$ w/w). All formulations were prepared 24 h before the study.

Following the preliminary in-vitro permeation studies, one of the anhydrous gels, namely PG:PEG300:ethanol (4:4:2 v/v), was selected for further study. Two emulsions (one oil-in-water, the other water-in-oil) were also prepared for the study. The emulsions were formulated in an attempt to reduce the applied dose of sitamaquine dihydrochloride and minimize its likely systemic uptake. The formulae of these additional preparations are shown in Table 2. In these formulations, a known amount of sitamaquine dihydrochloride was added. The anhydrous gels were formulated by first preparing a ternary solvent solution of PG, PEG300 and ethanol. The required amount of sitamaquine dihydrochloride was then added to this solvent system and left on a magnetic stirrer for 48 h to ensure dissolution of the drug. The oil-in-water (o/w) emulsions were prepared by first heating separately the oily and aqueous phases in separate containers to approximately 70°C. The SDS was added to the aqueous phase after the Pluronic F127 had completely dissolved. The oily phase was then added to the aqueous phase and mixed using a magnetic stirrer to ensure homogeneity of the cream base. The required amount of sitamaquine was dissolved in PG and this mixture added to the emulsion base. The water-in-silicone (w/s) emulsion was prepared by separately heating both aqueous and oily phases to approximately 60°C. The aqueous phase was then added to the oily phase and mixed using a magnetic stirrer to ensure homogeneity. All formulations were prepared 24 h before the study and stored at 2–8°C in glass borosilicate vials protected from light.

Table 1 Flux rates from sitamaquine dihydrochloride gels

Formulation	Human epidermal membrane (n = 6)		Full thickness BALB/c (n = 5)	
	Sitamaquine concentration in gel assayed by HPLC (% w/w)	Flux (Jss) ± s.e.m. ($\mu g\,cm^{-2}\,h^{-1}$)	Lag time ± s.e.m. (h)	Penetration rate ± s.e.m. ($\mu g\,cm^{-2}\,h^{-1}$)
PG:PEG300:ethanol (4:4:2 v/v)	11.33	0.058 ± 0.007	0.582 ± 0.147	0.173 ± 0.048
Ethanol:PC:IPM (5:4:1 v/v)	11.17	0.036 ± 0.002	1.515 ± 0.408	0.395 ± 0.043
PEG300:PG:PC (5:3:2 v/v)	10.81	0.069 ± 0.008	4.198 ± 1.636	1.009 ± 0.462
Water:PG:PEG400 (2:1:7 v/v)	29.77	0.047 ± 0.015	2.959 ± 1.103	0.608 ± 0.154

PG, propylene glycol; PEG300, polyethylene glycol 300; PC, propylene carbonate; IPM, isopropyl myristate; PEG400, polyethylene glycol 400.

Table 2 Formulary for sitamaquine dihydrochloride anhydrous gels and emulsions and the calculated % w/w and dose for each formulation

Formulation	Excipients	Formulated % w/w	
		5%	1%
Anhydrous gels	Ethanol	14.61	15.19
	Polyethylene glycol 300	41.68	43.34
	Propylene glycol	39.02	40.57
	Sitamaquine salt (actual)	4.68	0.90
	Calc. dose (μg)	322.17	71.52
		1%	0.5%
Oil-in-water emulsions	White soft paraffin	11.43	11.29
	Mineral oil	4.58	4.53
	Cetostearyl alcohol	6.18	6.10
	Water	26.34	26.02
	Poloxamer F127	0.92	0.91
	Sodium dodecyl sulfate	0.73	0.72
	Propylene glycol	48.83	46.01
	Sitamaquine salt (actual)	0.98	0.44
	Calc. dose (μg)	62.37	28.57
		1%	
Water-in-silicone emulsion	Cyclomethicone 5NF	10.02	
	Mineral oil	10.23	
	Dimethiconol blend 20	5.16	
	Silky wax 10	2.18	
	Emulsifier 10	2.13	
	Sodium chloride	1.01	
	Glycerine	3.08	
	Water	65.23	
	Sitamaquine salt (actual)	0.96	
	Calc. dose (μg)	60.15	

In-vitro skin permeation

Permeation studies, using Franz diffusion cells, were carried out using a previously published method (Howes et al 1996). The receptor compartment contained approximately 2.5 mL of phosphate-buffered saline (pH 7.4; PBS), which had been validated to ensure sink conditions over the time course of the study. The test formulations were applied to the surface of the skin in the donor compartment (time = 0) and samples of the receiver fluid samples were taken at predetermined time points and assayed for sitamaquine content using HPLC. Steady-state flux (infinite dose) or penetration rate (finite dose) was determined from the gradient of the linear portion of the curve obtained by plotting the cumulative amount of sitamaquine transported per unit area of skin against time for each formulation. The non-linear region of the curve represented the time prior to the establishment of steady-state diffusion, known as the lag time. Extrapolation of the linear portion of the curve to the x-intercept yielded the lag time.

Studies were performed to determine if sitamaquine dihydrochloride could penetrate full thickness female BALB/c mouse skin and human epidermal membranes (57-year-old female). When BALB/c mouse skin was used, a 25-μL volume of each gel was applied to the skin surface and receiver fluid samples were taken for HPLC analysis over 48 h. A total of five diffusion cells were used per formulation. When human epidermal membranes were used, a 100-μL volume was applied to the skin surface and gels were compared over 48 h using six diffusion cells per formulation. HPLC analysis was used to determine the sitamaquine concentration in each gel formulation (expressed as % w/w).

HPLC analysis

Analysis was carried out using a Dionex Summit HPLC system (PDA-100 photodiode array detector; ASI-100 automated sample injector; P580 pump) and Chromeleon chromatography software. The stationary phase consisted of a Hichrom (base deactivated silica) HI-5C18-250A column (250×4.6 mm, 5 μm ODS) and a pre-column guard (HI-5C18-10C). The mobile phase consisted of acetonitrile and water in a ratio of 28:72% v/v, with 0.1% v/v trifluoroacetic acid added to the final volume. The flow rate was set at 1 mL min^{-1} and an injection volume of 20 μL was used. The retention time was 5 min.

In-vivo leishmaniasis models

The in-vivo model for CL has previously been described (Kropf et al 1997). Briefly, mice were infected subcutaneously (0.2 mL) with 10^6–10^7 late-stage *L. major* JISH118 promastigotes. Cutaneous lesions were measured weekly using digital callipers (Jencons Leighton Buzzard, UK) and compared with the untreated control to evaluate therapy. To enable comparison of lesion sizes, a jittered analysis was applied to the data to separate superimposed points (Roberts et al 2000). Bodyweights were determined for each individual mouse throughout the study as a gross indicator of toxicity. Signs of skin irritation were graded according to OECD guidelines (Organisation for Economic Co-operation and Development; www.oecd.org), a scale used by both UK and US regulatory authorities.

The initial in-vivo study investigated the gels shown in Table 1. The gels were applied using a positive displacement Finnpipette. Cutaneous lesions appeared approximately 1 week after infection with 2×10^7 promastigotes. Mice were randomly allocated into groups of five per treatment group. Oral sitamaquine dihydrochloride was also tested at a daily dose of 10 mg kg^{-1} per mouse (prepared in PBS, pH 7.4). Daily dosing (topical formulations were applied as 20 mg cm$^{-2} \equiv$ 50 mg per day per mouse) was carried out from 11 days after infection and the study was completed on Day 28 after the start of treatment. Limiting dilution assays were carried out on lesion samples from remaining mice on the final day (Kropf et al 1997). The sitamaquine dihydrochloride gels were assayed by HPLC to determine percentage w/w concentrations.

The second in-vivo study investigated whether the emulsion type formulations could reduce toxicity and/or improve efficacy (see Table 2). A lower inoculum of 2.2×10^6 promastigotes was used to delay the progression of lesion growth to ulceration. The inoculum contained approximately 15% metacyclic promastigotes, selected by agglutination of peanut

agglutinin (da Silva & Sacks 1987). Mice were randomly allocated into groups of ten per treatment group. Daily dosing (topical formulations were applied as $15\,mg\,cm^{-2} \equiv 30$–40 mg/day/mouse) was carried out 10 days after infection and the study was completed on the Day 25. Parasite burden (from dermal scrapings) was determined using real-time PCR (Nicolas et al 2002). Real-time PCR analysis was carried out using an ABIPrism 7000 Sequence Detection System (Applied Biosystems, Warrington, UK) and the QuantiTect SYBR Green PCR kit Cat 204 143; Qiagen, Crawley, UK). Extraction of genomic DNA was carried out using a DNeasy tissue kit (Cat 69 504; Qiagen, UK). Three lesion samples of approximately 10–20 mg were taken for each mouse.

Radiolabelled drug uptake study

A known amount of radiolabelled sitamaquine dihydrochloride was added into each of the formulations listed in Table 2. This was achieved by first dissolving the ^{14}C-labelled sitamaquine dihydrochloride in ethanol and then allowing the desired aliquot to evaporate. The remaining ^{14}C-labelled sitamaquine was then mixed with the formulation containing the unlabelled drug. Three random samples were taken from each of the formulations containing radiolabelled drug for assay of the radiolabelled drug by liquid scintillation counting (LSC).

Sitamaquine penetration was measured using the finite dose method (Franz 1978). Approximately 5–10 mg of each formulation was accurately applied to the stratum corneum and 100-μL aliquots of the receiver fluid removed over 24 h and replaced with an equal volume of pre-warmed receptor fluid. At the end of 24 h, a mass balance study was performed to determine the amount of radiolabelled drug remaining in the following matrices: applicator, skin surface, stratum corneum and the remaining skin layer. The radiolabelled drug remaining on the skin surface was removed by swabbing with two cotton buds (previously soaked in a 1:1 v/v mixture of PBS:methanol) to remove unabsorbed drug and analysed by LSC. Radiolabelled drug remaining in the stratum corneum was determined by tape stripping using Scotch tape (3M), which was analysed by LSC. The remainder of the skin membrane was digested in Soluene-350 and an aliquot of the digest analysed by LSC. Percentage recovery from the total amount of applied radiolabelled drug was calculated for each individual Franz cell.

All samples (0.5 mL) were assayed in 4.5 mL Hionic-Fluor and counted using a Beckman LS 5000CE. A background correction was made for each aliquot by taking a receptor fluid sample from a Franz cell in contact with skin alone for the same time period. The amount of drug penetrating the skin was obtained by plotting the amount of sitamaquine penetrating per unit area against time. Values greater than 3 standard deviations from the mean were excluded in the analysis. Percentage recovery from the total amount of applied radiolabel was calculated for each individual Franz cell.

Binding studies

The binding of sitamaquine dihydrochloride to melanin and skin components was determined using a previously described method (Heard et al 2003). Briefly, for the binding to melanin study, 400 μL of a range of sitamaquine dihydrochloride solutions (namely 10 mM, 5 mM, 1 mM, 700 μM, 200 μM and 100 μM in PBS) were added to 2.5 mg of melanin powder and 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 $9000\,rev\,min^{-1}$ ($g = 4779$) 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 used for HPLC analysis and the resulting extract was assayed by HPLC.

To investigate the binding of sitamaquine dihydrochloride to skin components, BALB/c skin discs (full thickness) were delipidized as described by Wertz & Downing (1987). Weighed skin samples were placed in glass scintillation vials together with 1 mL of drug solution (sitamaquine dihydrochloride in PBS 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 $9000\,rev\,min^{-1}$ 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 pooled samples were assayed by HPLC. The results were graphed as μg per mg tissue versus the initial incubation concentration.

Statistical methods

For the in-vitro skin study, statistical analysis of the different sitamaquine gel formulations (PG:PEG300:ethanol, ethanol:PC:IPM, PEG300:PG:PC, water:PG:PEG400) on the amount of sitamaquine penetrating human epidermal membranes at each time point was performed using a one-way analysis of variance (Table 1). One-way analysis of variance with Tukey Kramer multiple comparison tests was performed using GraphPad InStat version 3.05 for Windows 95/NT (GraphPad Software, San Diego CA USA). Statistical analysis of the different sitamaquine formulations (o/w emulsion, w/s emulsion, gel) on the amount of sitamaquine penetrating full thickness BALB/c skin at each time point was also performed using a one-way analysis of variance with Tukey Kramer multiple comparison tests.

For the second in-vivo study, statistical analysis of parasite burden to compare the treatment groups with the untreated control, was performed using a one-way analysis of variance (GraphPad InStat). A Dunnett's multiple comparison post-test was used. The Kolmogorov-Smirnov test was used to ensure normal distribution of data. Results were considered significant when $P < 0.05$.

For the binding studies, statistical analysis of the amount of bound sitamaquine was used to compare the delipidized skin with normal skin using a paired two tailed t-test (GraphPad InStat).

Results

Initial studies investigated the activity of sitamaquine dihydrochloride using two in-vitro *Leishmania* models.

Table 3 ED50 values in μM at 72 h for promastigotes at 26°C (95% confidence intervals)

Compounds	N	Sitamaquine dihydrochloride	Amphotericin B (control)	Sensitivity
Leishmania aethiopica Khartoum	1	31.455 (31.191–31.719)	0.022 (0.013–0.031)	20–27
	2	75.727 (72.520–78.934)	0.079 (0.058–0.101)	20–22
	3	53.583 (48.494–58.572)	0.058 (0.054–0.062)	26–30
Leishmania major JISH118	1	41.957 (40.039–43.874)	0.104 (0.098–0.110)	11–18
	2	66.564 (52.867–80.261)	0.324 (0.322–0.326)	21–24
	3	28.322 (25.140–31.504)	0.119 (0.108–0.129)	15–17
	4	8.567 (6.940–10.194)	0.093 (0.068–0.118)	27–31
Leishmania mexicana LV4	1	13.967 (12.526–15.409)	0.096 (0.072–0.120)	14–20
	2	15.032 (12.018–18.046)	0.123 (0.091–0.156)	29–35
	3	30.869 (28.415–33.323)	0.124 (0.118–0.130)	32–35
Leishmania mexicana BEL21	1	14.124 (2.348–25.901)	0.043 (0.036–0.051)	25–27
	2	5.726 (3.720–7.733)	0.021 (0.020–0.022)	15–19
	3	6.098 (5.147–7.049)	0.019 (0.018–0.019)	15–17
Leishmania panamensis Boynton	1	28.780 (27.356–30.204)	0.083 (0.076–0.089)	14–17
	2	40.181 (38.316–42.006)	0.069 (0.059–0.078)	19–27
	3	36.580 (28.284–44.877)	0.109 (0.108–0.110)	10–12
Leishmania amazonensis LV81	1	30.188 (26.377–32.000)	0.104 (0.097–0.111)	32–36
	2	37.499 (36.226–38.772)	0.098 (0.080–0.117)	34–37
	3	25.829 (21.705–29.953)	0.077 (0.067–0.087)	27–31
Leishmania donovani HU3	1	23.011 (16.996–29.025)	0.038 (0.037–0.038)	14–16
	2	29.187 (24.228–34.146)	0.042 (0.025–0.059)	9–10
	3	39.945 (38.119–41.772)	0.052 (0.025–0.078)	8–8

N, experiment number. Sensitivity refers to the fluorescent response/background signal.

In the promastigote assay, sitamaquine dihydrochloride was found to exhibit varying activity against the range of species tested (5.7 $-75.7\,\mu$M) (Table 3). In comparison with the control drug, amphotericin B, which was active in the nanomolar range for all species, sitamaquine dihydrochloride was about 100-times less active. ED50 values of sitamaquine dihydrochloride against both the *L. mexicana* strains were in the low micromolar range. In the amastigote assay, sitamaquine dihydrochloride was active against all the species tested over the range of 2.9 to 18.9 μM (Table 4). In this assay, sodium stibogluconate was used as the control drug. However, the ED50 for sodium stibogluconate against several species in this 3-day assay was greater than the concentrations tested. As an indicator of toxicity, any reductions in the number and morphology of the Mϕ were recorded for each drug at each of the concentrations tested. On the basis of this assay, sitamaquine dihydrochloride appeared to be moderately toxic at the highest concentrations tested (30 μM).

The preliminary in-vitro skin permeation studies demonstrated that sitamaquine dihydrochloride when formulated in the form of either anhydrous or hydrous gels (Table 1) readily penetrated full thickness mouse skin, and to a lesser extent human epidermal membranes. A gel formulation was initially chosen because the use of minimum solvents could ensure the drug concentration was all in solution but close to the solubility limit and therefore maintained optimal partitioning into the skin. The ranking of the order of penetration rate across BALB/c skin was PEG300:PG:PC > water:PG:PEG400 > ethanol:PC:IPM > PG:PEG300:ethanol, although there was some variability between Franz cells. The penetration rate of sitamaquine dihydrochloride across BALB/c skin ranged from $1.009 \pm 0.462\,\mu g\,cm^{-2}\,h^{-1}$ for the PEG300:PG:PC gel, to $0.173 \pm 0.048\,\mu g\,cm^{-2}\,h^{-1}$ for the PG:PEG300:ethanol gel. Sitamaquine dihydrochloride penetration from the PEG300:PG:PC gel was approximately 5.8-times greater than from the PG:PEG300:ethanol gel, 2.6-times greater than the ethanol:PC:IPM gel, and just over 1-times greater than the water:PG:PEG400 gel. The lag times observed ranged from 0.582 ± 0.147 to 4.198 ± 1.636 h. These results suggest that the various formulations and not the BALB/c skin were limiting transport across the skin. The calculated penetration rates across human epidermal membranes indicated the general rank order for penetration rate for the various gels: PEG300:PG:PC > PG:PEG300:ethanol > water:PG:PEG400 > ethanol:PC:IPM, with a penetration rate of $0.069 \pm 0.008\,\mu g\,cm^{-2}\,h^{-1}$ being obtained for the gel prepared with PEG300:PG:PC, $0.058 \pm 0.007\,\mu g\,cm^{-2}\,h^{-1}$ for the PG:PEG300:ethanol gel, $0.036 \pm 0.002\,\mu g\,cm^{-2}\,h^{-1}$ for the ethanol:PC:IPM gel, and $0.047 \pm 0.015\,\mu g\,cm^{-2}\,h^{-1}$ for the water:PG:PEG400 formulation. Surprisingly, no lag times were observed for sitamaquine dihydrochloride across human epidermal membranes. However a one-way analysis of variance test did not show any significant difference between the amounts penetrating from the various gels ($P > 0.05$; Table 1), suggesting that the human epidermal membrane and not the gels was limiting the transport of sitamaquine dihydrochloride across the human skin. When comparing the results of the two studies, there was a 3- to 15-fold increase in the penetration rate of sitamaquine dihydrochloride across murine skin as opposed to human skin. Furthermore, although there was a definite difference in penetration rate of sitamaquine dihydrochloride across murine skin from the various formulations, there was no

difference in the penetration rate of sitamaquine dihydrochloride from the gels across human skin. This result is not surprising as animal skin is thought to be more readily permeable to compounds than human skin (Howes et al 1996) due to species differences in composition, thickness and number of hair follicles. It should be noted that while both skin membranes were approximately 1–2 mm thick, the human epidermal membrane did not contain a dermal layer whereas the BALB/c skin was full thickness.

Since the preliminary in-vitro studies established that sitamaquine dihydrochloride could readily penetrate BALB/c skin, an initial in-vivo study was performed using a dose of 50 mg gel/mouse/day to determine if the various gels containing sitamaquine dihydrochloride (gel concentration ranged from 11 to 18% w/w) exhibited any efficacy and/or toxicity. However, after only three days of treatment with the various gels, the BALB/c mice exhibited signs of toxicity (namely horripilation, weight loss, hunched body). Only four doses were applied since six mice had died on the fourth day (final day of treatment) and a further 12 mice had died by the eleventh day. The data obtained for this study have not been presented. As the least toxic gel was found to be prepared using PG:PEG300:ethanol, it was re-formulated at a lower strength (5 and 1% w/w). In addition, two emulsion formulations were also developed in an attempt to reduce toxicity (Table 2). All the new formulations were well tolerated, except for a small amount of skin irritation caused by the o/w emulsion, possibly due to the high concentration of PG and/or the presence of the anionic surfactant SDS. According to OECD guidelines, the skin irritation was graded as follows: erythema as 1, −2, and oedema as 1. In no case was there any significant change in the bodyweight of the mice ($<1\%$ change in mean bodyweight). Unfortunately, however, none of the formulations showed activity. For example, a jittered scatterplot (Figure 1) indicated that none of the formulations exhibited any decrease in mean lesion diameter. In addition, a one-way analysis of variance (performed at Day 15, end of treatment) using the Dunnett's multiple comparison test (GraphPad InStat), demonstrated no significant difference in parasite burden between the sitamaquine dihydrochloride treated groups and the untreated control ($P = 0.7747$).

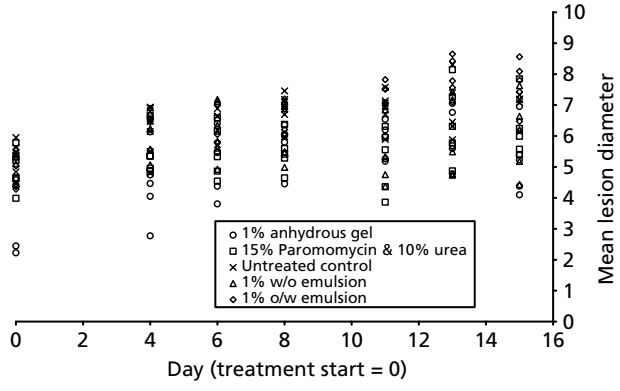

Figure 1 Jittered scatterplot of effect of treatment duration on mean lesion diameter for *Leishmania major* (n = 10).

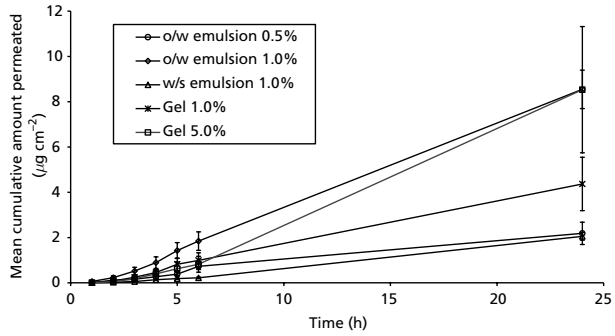

Figure 2 Mean cumulative ^{14}C-sitamaquine amount across full thickness BALB/c skin (n = 6 ± s.e.m.).

These sitamaquine dihydrochloride formulations were then tested in a radiolabelled study to investigate skin uptake. The amount of sitamaquine dihydrochloride present in each formulation is shown in Table 2; the mean cumulative quantity penetrating BALB/c skin per unit area is shown in Figure 2. Comparison of the emulsions at a similar percentage concentration indicates a greater cumulative amount per unit area with the o/w formulation. The 1% o/w emulsion had a significantly greater mean cumulative amount of sitamaquine penetration compared with the 1% w/s emulsion ($P < 0.05$, one-way analysis of variance). The w/s emulsion had the lowest cumulative amount penetrating per unit area. By 24 h after application, the greatest percentage penetration of the radiolabel was from the 1% anhydrous gel and 1% o/w emulsion, giving $10.96 \pm 3.66\%$ and $9.89 \pm 0.79\%$, respectively (Figure 3). Percentage retention in the stratum corneum strippings and remaining skin membrane suggest the o/w emulsion had the greatest retention, particularly in the tape-stripped skin membrane. The 1% gel appeared to have a higher percentage penetration at 24 h and skin retention than the 5% gel. The w/s emulsion showed the lowest percentage penetration and skin retention, along with the 5% gel. This confirmed that the sitamaquine radiolabel was penetrating and being retained in BALB/c skin in this in-vitro model.

For the binding studies, sitamaquine dihydrochloride was shown to bind melanin (*Sepia officinalis*) in a

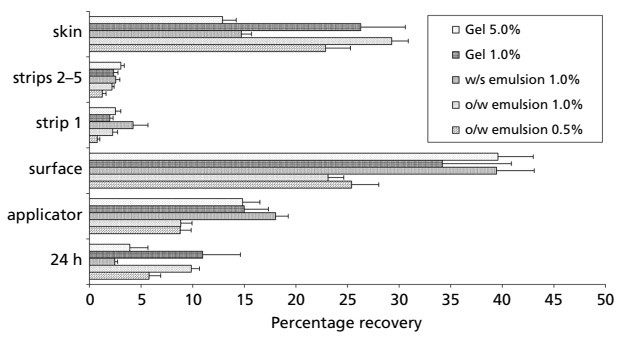

Figure 3 Mean percentage recovery for various sitamaquine dihydrochloride formulations (n = 6 ± s.e.m.).

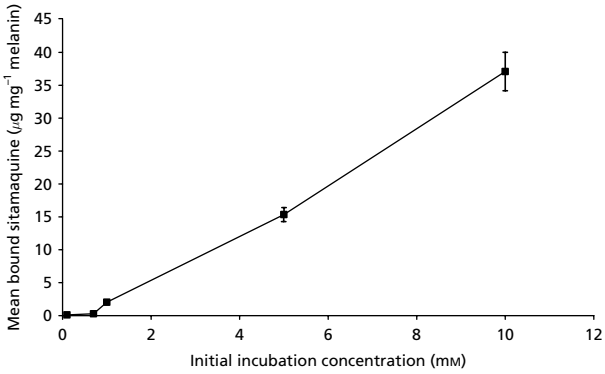

Figure 4 Binding of sitamaquine to melanin (*Sepia officinalis*) as a function of the initial concentration incubated (mean ± s.e.m., n = 3).

dose-dependent manner (Figure 4). Sitamaquine dihydrochloride contains several basic amine groups that may interact with hydroxyl melanin groups. Melanin affinity for amine compounds is also known to be very high. Sitamaquine dihydrochloride binding to delipidized BALB/c skin was significantly greater than that binding to normal skin ($P < 0.0001$, two tailed *t*-test), based on a weight-to-weight comparison (Figure 5).

Discussion

The preliminary studies investigating the activity of sitamaquine dihydrochloride against *Leishmania* species were performed to determine the intrinsic activity against the parasite. Although sitamaquine dihydrochloride activity against both promastigotes and amastigotes was investigated, it is the activity against the amastigote that is clinically relevant. In the promastigote assay, *L. mexicana* appeared particularly susceptible to sitamaquine dihydrochloride, although differences in metabolism and proliferation must be considered. Differences in fluorescent signal were seen between species, which probably reflects the rate/extent of Alamar Blue uptake or metabolism and cell number/size (reflected in sensitivity), rather than intrinsic sensitivity to drugs. As a control, both sitamaquine dihydrochloride and amphotericin B were incubated in media alone and fluorescent readings were taken after 24 h incubation. Neither sitamaquine dihydrochloride nor amphotericin B reduced the dye.

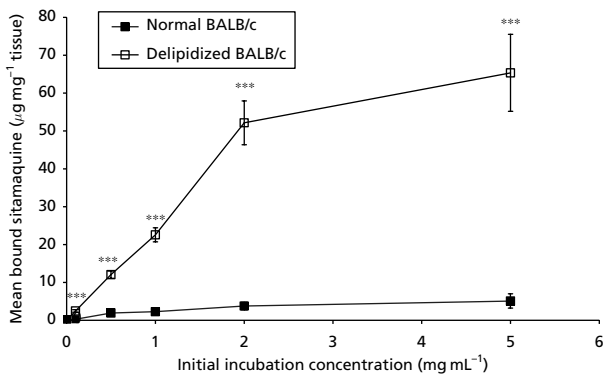

Figure 5 Sitamaquine binding to full thickness BALB/c skin (native and delipidized) (mean ± s.e.m., n = 4). ***$P < 0.0001$, two tailed *t*-test.

In the amastigote assay, sitamaquine dihydrochloride had lower ED50 values than those against promastigotes for most of the species tested (*L. donovani*, *L. panamensis*, *L. major* and *L. aethiopica*). Values obtained in this study were similar to previously reported results (Berman & Lee 1983; Neal et al 1985; Callahan et al 1997), however differences in experimental conditions must be taken into account, such as Mϕ type, incubation time and % infection. In this study, the ED50 values determined for sitamaquine dihydrochloride against the CL species tested ranged from 2.1 to 18.9 μM (equivalent to 0.9–7.9 $\mu g\,mL^{-1}$), as shown in Table 4. Previous in-vitro studies using amastigotes infected with CL species found ED50 values of 1.2–5.0 μM (equivalent to 0.5–2.1 $\mu g\,mL^{-1}$ for sitamaquine dihydrochloride) (Berman & Lee 1984). In addition, the same researchers reported that the ED50 value of sitamaquine dihydrochloride against *L. donovani* was approximately 9.7 μM (equivalent to 4.0 $\mu g\,mL^{-1}$). Sitamaquine dihydrochloride activity (expressed as an ED50) against *L. donovani* after 7 days has been reported to be 1.4 μM (equivalent to 0.59 $\mu g\,mL^{-1}$). Evidence of Mϕ toxicity was seen at a concentration of 7.2–64.8 μM (equivalent to 3–27 $\mu g\,mL^{-1}$) (Neal & Croft 1984). Also, both *L. amazonensis* and *L. mexicana* had a high number of amastigotes per Mϕ in the untreated control, which might contribute to the poor activity over the concentration range tested. ED50 values for sodium stibogluconate could not be determined for several species, including *L. mexicana*, *L. major* and *L. amazonensis*. The pentavalent antimonials, such as sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) have been the mainstay of therapy for leishmaniasis since the 1940s (Berman 2005). This lack of in-vitro activity has been documented previously (Berman & Wyler 1980; Sereno & Lemesre 1997). Pentostam is known to have improved activity in a 5-day assay (Neal & Croft 1984). Serum concentrations of Pentostam and amphotericin B have been reported as 19–41 $\mu g\ Sb\,mL^{-1}$ and 1 μM, respectively (Sereno & Lemesre 1997; Frezard et al 2001).

Studies using the in-vitro Franz diffusion cell confirmed that sitamaquine dihydrochloride penetrated both human epidermal and full thickness BALB/c skin from a range of gels. Skin penetration can occur via the transappendageal or transepidermal route, although the latter is regarded as the major pathway for most permeants (Bronaugh & Maibach 1999). The transport of drugs by the transepidermal route has been shown to occur predominantly by passive intercellular diffusion. The gels showed a different order of penetration rate between mouse and human skin, with sitamaquine dihydrochloride penetrating the former at a greater rate from all formulations of the drug. In general, animal skin is known to be more permeable to compounds due to differences in composition and skin structure between the two species. For example, in human skin, melanocytes are associated with the junction between the epidermis and dermis, whereas they are predominantly found within hair follicles in the

Table 4 ED50 values in μM (95% confidence intervals) at 72 h for cutaneous lesions amastigotes in macrophages (Mϕs) at 34°C and *Leishmania donovani* HU3 amastigotes in Mϕs at 37°C

Parasite species	N	Sitamaquine dihydrochloride	Sb^v (control)	% Infected Mϕs (0 h)	% Infected Mϕs (72 h)	Mean N° amastigotes/Mϕ
Leishmania aethiopica Khartoum	1	2.896 (2.274–3.517)	ND	78.25 (s.d. = 7.14)	88.25 (s.d. = 5.68)	12.75 (s.d. = 12.55)
	2	15.382 (11.123–19.640)	ND	86.00 (s.d. = 7.79)	98.75 (s.d. = 1.26)	60.00 (s.d. = 34.22)
Leishmania major JISH188	1	4.456 (3.821–5.091)	ND	76.75 (s.d. = 4.79)	82.0 (s.d. = 8.41)	23.15 (s.d. = 17.41)
	2	5.334 (4.562–6.106)	ND	76.75 (s.d. = 4.79)	82.0 (s.d. = 8.41)	23.15 (s.d. = 17.41)
	3	14.509 (13.4473–15.5710)	ND	94.50 (s.d. = 2.13)	98.00 (s.d. = 1.83)	23.85 (s.d. = 11.28)
Leishmania mexicana LV4	1	ND	ND	90.00 (s.d. = 2.83)	97.25 (s.d. = 1.71)	71.25 (s.d. = 23.00)
	2	18.907 (11.392–26.421)	ND	95.25 (s.d. = 4.86)	100.00 (s.d. = 0.00)	76.20 (s.d. = 25.90)
Leishmania panamensis Boynton	1	2.091 (1.636–2.545)	0.794 (0.301–1.287)	89.50 (s.d. = 3.87)	93.50 (s.d. = 3.70)	19.25 (s.d. = 19.53)
	2	5.499 (4.679–6.319)	25.844 (0.066–51.622)	89.00 (s.d. = 1.49)	91.75 (s.d. = 1.71)	25.50 (s.d. = 11.82)
	3					
Leishmania amazonensis LV81	1	ND	ND	95.5 (s.d. = 0.71)	96.75 (s.d. = 3.77)	84.85 (s.d. = 21.99)
	2					
	3					
Leishmania donovani HU3	1	10.58 (9.63–11.53)	ND	94.75 (s.d. = 2.22)	98.50 (s.d. = 0.58)	82.50 (s.d. = 20.70)
	2	ND	23.04 (15.41–30.67)	99.00 (s.d. = 0.82)	99.50 (s.d. = 1.00)	82.50 (s.d. = 20.70)
	3	8.76 (7.72–9.80)	ND	94.75 (s.d. = 2.22)	98.50 (s.d. = 0.58)	45.55 (s.d. = 29.51)

N, experiment number; Sb^v, pentavalent antimony (sodium stibogluconate); ND, ED50 values could not be calculated within the concentration ranges tested. Concentrations for Sb^v are expressed as μg Sb^v L^{-1} as the molecular weight of sodium stibogluconate is unknown.

mouse. Mouse skin also contains a much greater number of hair follicles and therefore drugs penetrating via the transappendageal route might have a greater penetration rate through mouse skin compared with human skin. The effect of formulation excipients on skin type will therefore differ, since each excipient will interact differently with each skin type. For example, an excipient that increases permeation via disruption of the lipid bilayers might be expected to have a greater impact on skin containing a higher percentage of lipids. However, the excipient effect will also depend on the particular route of penetration. In addition, there was no significant difference in the penetration of sitamaquine dihydrochloride across human skin ($P > 0.05$, one-way analysis of variance). This suggests that the human epidermal membrane was the limiting factor in penetration rate and not the gel formulations.

The initial in-vivo study highlighted problems of toxicity with these gel formulations, which would suggest transdermal delivery in the mouse model. Since the *Leishmania* parasites are found within infected dermal Mϕs, localized delivery to infected tissues rather than uptake into the systemic circulation is desired. Our preliminary in-vitro studies confirmed that sitamaquine dihydrochloride from the gel formulations readily penetrated full thickness BALB/c skin (Table 1). In an attempt to retain the drug within the skin, the drug was reformulated as a gel containing a lower concentration of drug and both o/w and w/o emulsions. The w/o type emulsion was of particular interest as it was felt that this formulation would delay the skin penetration of the drug. Sitamaquine dihydrochloride is very hydrophilic. It would be expected to predominantly partition into the internal aqueous phase of the water-in-oil emulsion. Skin penetration depends partly on diffusion within the vehicle prior to skin partitioning, and the external oleaginous phase would slow the diffusion of the internally dispersed aqueous phase. In-vivo, these emulsions were found to be less toxic compared with the original gels. Unfortunately, no reduction of lesion size or parasite burden was observed, indicating that the drug formulated in this manner did not exhibit any significant efficacy. The reduced toxicity observed with this formulation suggests a lower systemic uptake, probably partially resulting from the decreased dose of drug and partially from a slower release rate.

To further examine the fate of sitamaquine dihydrochloride skin uptake, a radiolabelled study was performed. Both the 24-h penetration profile and skin recovery results confirmed that, perhaps not surprisingly, out of the three formulations, the o/w emulsion showed the greatest drug penetration across full thickness BALB/c skin (Figure 2). In contrast the w/s formulation produced a slower drug penetration, which was probably due to a delayed release from the internal water phase of this formulation. The o/w formulation contained approximately 26% water compared with 65% for the w/s formulation and this may have contributed to a greater drug thermodynamic activity (driving force for drug absorption) for the former. However, the effect of other excipients should also have to be taken into account as these may alter the thermodynamic activity of the drug within the vehicle and reduce flux. The low water content and oleaginous excipients (white soft paraffin, mineral oil) for the o/w cream are likely to create occlusion and skin hydration is known to enhance absorption. The o/w emulsion also showed the greatest drug retention in the skin, as determined by tape stripping. This type of formulation might therefore be more suitable for delivering sitamaquine dihydrochloride to the infected Mϕs. However, the tape stripping technique used in this radiolabelled study is only an estimate for drug retention within the outermost skin layers. In theory, tape stripping removes the stratum corneum, which is a rate-limiting barrier to absorption and is known to act as a reservoir for many chemicals. An important consideration in tape stripping is that the vehicle components may alter keratinocyte cohesion (Surber et al 1999). Therefore, depending on the formulation, tape stripping may remove different amounts of skin due to variations in cell adhesion. Tape strips may also remove skin from various depths (depending on the pressure applied, wrinkles, skin condition). Nevertheless, the radiolabelled study confirmed sitamaquine was being retained within the mouse skin. However, the lack of efficacy in-vivo would suggest that the required sitamaquine dihydrochloride concentrations were not being retained for a sufficient time within the infected Mϕs. An estimate, based on the in-vitro studies, suggests a minimum concentration of 1–20 μM for at least 3 days would be necessary to kill parasites.

Due to the toxicity and lack of efficacy seen in-vivo, skin binding studies were carried out to investigate the preferential affinity for sitamaquine dihydrochloride between untreated native and delipidized BALB/c skin and therefore the likely distribution within skin components. Binding studies with native and delipidized skin showed sitamaquine dihydrochloride to bind preferentially to delipidized BALB/c skin. This was expected since sitamaquine dihydrochloride is a salt and a charged molecule. Drug binding within the delipidized skin may occur to keratin, melanin and other skin proteins, although there are also covalently bonded lipids that are not removed by the delipidization process (Heard et al 2003). Removal of non-covalently bound lipids may allow greater access to binding sites within the essentially proteinaceous skin components. For very hydrophilic drugs such as sitamaquine dihydrochloride, the intercellular lipids of the stratum corneum can provide much resistance to skin penetration. The preferential binding of sitamaquine dihydrochloride to protein components within the skin may explain the skin retention found in the radiolabelled study. However, protein binding might also reduce the effective concentrations available to penetrate infected dermal Mϕs. Several antimalarials (e.g. the 4-aminoquinolines chloroquine and amodiaquine; 8-aminoquinolines primaquine and pamaquine) are known to bind to melanin (Sjolin-Forsberg et al 1996; Banning & Heard 2002). Sitamaquine dihydrochloride contains several amine groups, which are known to have a high affinity for melanin (Fukuda et al 2000). Accumulation, which may occur in melanin-rich tissues (e.g. eyes, hair, skin), could account for the associated side-effects such as visual disturbances, hair depigmentation/loss and skin reactions (Petty et al 1999). As such, knowledge of

melanin binding may therefore indicate whether a drug is likely to accumulate in tissues. In-vitro binding studies indicated that sitamaquine dihydrochloride bound melanin in a dose-dependent manner. Such drug–protein binding may explain the retention of drug in the skin observed in this study; however, it might also result in a reduction of the amount of drug reaching the underlying skin layers and thus the infected dermal Mϕs. As the intrinsic activity of the drug has been confirmed, such a hypothesis may help to explain the lack of efficacy in the in-vivo BALB/c studies.

Sitamaquine dihydrochloride metabolism and/or elimination in the mouse model might also explain its lack of efficacy. Although extensive metabolism of sitamaquine dihydrochloride is known to occur following oral administration (Theoharides et al 1985; Sherwood et al 1994; Petty et al 1999; Dietze et al 2001), it is unlikely to occur in this case. The skin does contain many of the enzymes present in the liver; however, the metabolic capability is much lower (Hotchkiss 1998). The lack of efficacy after topical delivery of this molecule (and other non-enteral routes) is possibly due to inadequate skin metabolism to a more active compound. Oral efficacy may depend on metabolic derivatives after first pass metabolism. Sitamaquine dihydrochloride has been shown to have intrinsic activity against the *Leishmania* parasite in-vitro; however, its in-vivo metabolites are possibly more potent antileishmanials. The differences in metabolic handling between species might also explain the ambiguous efficacy and toxicity results both in human and animal studies. In general, it is known that the 8-aminoquinolines (including sitamaquine & primaquine) are more active against mouse models of VL than CL (Peters et al 1980).

Both the radiolabelled uptake study and the skin binding study confirmed sitamaquine dihydrochloride was being retained within the BALB/c skin. However, despite in-vitro parasite studies confirming intrinsic activity of sitamaquine dihydrochloride against the parasite, the in-vivo results were disappointing. The lack of efficacy in the mouse model might be owing to inadequate uptake of sitamaquine dihydrochloride (or its metabolites) into the dermal Mϕs. To determine this, it would be necessary to further investigate Mϕ uptake. It would also be interesting to test metabolites of sitamaquine dihydrochloride in both in-vitro and in-vivo models of cutaneous leishmaniasis.

Conclusion

Although sitamaquine dihydrochloride was shown in-vitro to have activity against a range of CL species, the in-vivo studies demonstrated neither a reduction in lesion progression nor in parasite burden. However, both the binding and radiolabelled studies confirmed skin uptake and retention. The lack of efficacy suggests that the drug (or more active metabolites) is not being delivered to the infected dermal Mϕs at the required concentration and/or rate. Further development of sitamaquine topical formulations for the treatment of CL should investigate both Mϕ uptake and the possibility of more potent metabolites.

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