Informative title: Drug permeation and barrier damage in *Leishmania*-infected mouse skin

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Synopsis

Objectives: Pathological disorder can disrupt the barrier integrity of the skin thereby altering the drug delivery from topical formulations to the target site. Cutaneous leishmaniasis is an infection of the dermal layers of the skin, and manifests as a variety of skin lesions from defined nodular forms to plaques and chronic ulcers. The aim of this work was to characterise the physiology and barrier integrity of the *Leishmania*-infected BALB/c mouse skin and how they impacted delivery of drugs to the skin.

Methods: A histological evaluation of the structural differences between uninfected and infected skin was performed using an H&E, elastic Von Gieson and IBA-1 stain. As a CL nodule developed and progressed, the skin pH, hydration and transepidermal water loss were recorded. Finally, Franz diffusion cells were used to evaluate the influence of the infection on drug delivery through the skin.

Results: We found (i) structural changes in both the epidermal and dermal layers due to the ingress of inflammatory cells as shown by immunohistochemistry, (ii) a significant increase in trans-epidermal water loss (TEWL) and (iii) a significantly higher permeation of model permeants, caffeine and ibuprofen and the anti-leishmanial drugs buparvaquone and paromomycin, for *Leishmania*-infected skin compared to uninfected skin. The infection had no measurable influence on skin pH and hydration.

Conclusions: we report profound changes in the skin barrier physiology, function and permeability to drugs of *Leishmania*-infected skin.

Introduction

Cutaneous leishmaniasis (CL) is a parasitic disease that mainly affects poor communities in low- and middle-income countries with approximately 10 million people affected. [1] CL is characterized by a variety of clinical symptoms ranging from defined nodular forms to chronic ulcerative lesions. Non-complicated lesions tend to heal spontaneously, a process that can take 3 to 18 months depending on the *Leishmania* species and host susceptibility factors, and nearly always leave a disfiguring scar. [2] Available treatments such as miltefosine, amphotericin B and pentavalent antimonials are constrained by long treatment courses, parenteral drug administration, toxicity, cost and variable efficacy.

In CL, *Leishmania* parasites typically reside in macrophages in the dermal layer of the skin. Although most current treatments for leishmaniasis are administered by other routes, for simple CL infected tissue can potentially be reached by topically applied drugs. Topical application is advantageous as it allows local and targeted drug exposure, thereby reducing the potential side effects and the need to carefully monitor the patient when given systemic treatment. Moreover, the ease of application makes it patient-friendly. Several topical formulations have been developed in the past to the point of clinical trials and used to treat CL infections, for example, the aminoglycoside paromomycin sulphate (WR279,397, [3] another paromomycin formulation Leshcutan® (Teva, Israel), and amphotericin B. [4] None of these proved to be entirely satisfactory due to side effects or lack of efficacy. For example local side

effects such as burning and irritation of the skin were commonly reported upon application of Leshcutan®. [5, 6] This is due to the presence of 12% methylbenzethonium chloride, a cationic quaternary ammonium compound that facilitates drug permeation into the skin. [7, 8]

The success of a topical formulation to treat CL depends on the anti-leishmanial potency of the active ingredient and the amount of drug that reaches the parasites. [9] Skin disorders can alter barrier integrity resulting in increased or decreased drug delivery from topical formulations to their target site in the skin. [10-12] So far, drug delivery to diseased skin has mainly focused on non-infectious diseases, for example, psoriasis. In psoriatic plaques a reduced barrier integrity resulted in increased drug permeation compared to healthy skin. [13, 14] Infectious skin diseases caused by pathogens such as herpes, *Tinea* and *Candida albicans* have also been found to disrupt the physiology and barrier function of the skin. The barrier integrity in tinea-affected skin, was characterised by reduced stratum corneum hydration, enhanced proliferation and disturbed differentiation of the epidermis compared to healthy skin. [11]

To date there is no information available on the permeability of *Leishmania*-infected skin. An understanding of the permeability of skin infected by *Leishmania* parasites will help the optimisation of drug delivery. Thus, we aimed to characterise the skin barrier function in the early stages of CL in a mouse model with respect to histology, trans-epidermal water loss (TEWL), skin pH, and the permeability to topically applied compounds. Caffeine and ibuprofen, two model permeants [15, 16] were chosen for study because of their divergent physico-chemical

properties. Paromomycin sulphate was selected because it is the only currently available topical drug for CL while amphotericin B and buparvaquone were selected as attempts to reformulate these molecules [17] (http://www.dndi.org/diseases-projects/portfolio/anfoleish-cl.html, accessed 3-06-2015) into topical formulations have been reported.

Material and Methods

Parasite strain and animals

Leishmania major (MHOM/SA/85/JISH118) promastigotes were maintained in Schneider's insect medium (Sigma Aldrich, UK) supplemented with 10% heat-inactivated foetal calf serum (Harlan, UK) at 26°C. The parasites were routinely passaged through BALB/c mice and low passage number promastigotes (typically below passage number 3) were used for infection. Female BALB/c mice of about 6-8 weeks old, were purchased from Charles River (Margate, UK) and housed in a controlled environment of 55% relative humidity and 26°C. They were provided with tap water and a standard laboratory diet ad libitum. All *in vivo* experiments were carried out under license (PPL 70/6997) at the London School of Hygiene & Tropical Medicine (LSHTM) after discussion with the veterinarian, clearance through the LSHTM research ethics committee and according to UK Home Office regulations.

In vivo CL model

Five female BALB/c mice (6-8 weeks old; Charles River Ltd., UK) were shaved and injected the following day with $2x10^7$ stationary phase *L.*

major JISH118 promastigotes (200 µL) subcutaneously on the rump above the tail. Three control mice were injected with medium that did not contain parasites.

After about 7 days, a small papule at the site of injection was visible in the infected mice. The lesion size was measured daily using callipers. TEWL, skin pH and hydration were measured every other day as follows: mice were left to acclimatise in the room for 1 hour before being placed in the induction chamber. Anaesthesia were induced at 3% isoflurane with 100% oxygen at a flow rate of 2.5 L/min until no movement was observed and maintained with 2% isoflurane and 100% oxygen at 2.5 L/min. TEWL was measured using the Aquaflux AF102 (Biox, London), the equipment was calibrated according to the manufacturer's guidelines prior to starting the experiment and was baseline calibrated prior to use each day. Skin hydration was measured using the moisture checker (MY-808S, Scalar, Japan) and the skin-pH was obtained with the Skin-pH-Meter® PH 905 from CK electronics (Köln, Germany). The mice recovered from anaesthetics on a warm plate (37˚C). Statistical analysis were performed using SPSS software version 19.0.

When the lesion diameter reached 5-6 mm, the mice were sacrificed and the papule was excised for histological examination or permeation studies. For histological evaluation, the samples were collected and fixed in 10% neutral formalin solutions until they were further processed at the UCL Institute of Neurology (Queen Square, London). Images of the processed and stained samples were taken using a Leica DMRB

microscope (Leica, Germany) equipped with a Leica DFC 420 camera controlled by Leica Application Suite (version 3.1). For permeation studies, the uninfected control tissue was taken from above the CL nodule on the back of the same donor mouse.

Investigation into permeability of infected vs uninfected skin

Franz cell devices with narrow diameter (5 mm) were produced (Soham Scientific, Fordham, UK) in order to measure the permeation of a drug through a *Leishmania* infected skin area (5 mm diameter). All drugs were purchased from Sigma Aldrich (Dorset, UK) except for amphotericin B, which was obtained from VWR International (Leicestershire, UK) and [^{3}H]-paromomycin sulphate (specific activity: 0.2 Ci/mmol, radiochemical purity: 99.8% and radioactive concentration: 500 µCi/mL) was obtained from Moravek Biochemicals (Brea, CA). Saturated donor solutions for caffeine, ibuprofen, paromomycin sulphate and buparvaquone were prepared by adding an excess amount of drug to PBS (Sigma Aldrich) and leaving it to stir at 32 ˚C for 48 hours prior to the experiment. The paromomycin sulphate drug suspension was centrifuged at 13000rpm for 30 minutes and the supernatant was removed and spiked with 10 µCi of [3H]-paromomycin sulfate per Franz diffusion cell. The amphotericin B donor solution was prepared by adding an excess amount of drug to PBS followed by 1 hour of sonication.

The full-thickness skin pieces were inspected for macroscopic damage and muscle and fat tissue were carefully removed using forceps. Skin pieces were cut in circular discs of about 2cm diameter. They were then

mounted between the donor and receptor compartment of the Franz cell device and kept in place by a clamp. The receptor fluid consisted of a PBS solution that was sonicated for 30 minutes to remove air bubbles prior to adding it to the Franz cell device. For the permeation of the more lipophilic compounds, buparvaquone and amphotericin B, PBS was supplemented with 2% hydroxypropyl-β-cyclodextrin (Sigma Aldrich) [17] (CD) in order to ensure sink conditions during the experiment. A small magnetic stirrer was introduced through the sampling arm, taking care not to introduce bubbles. Prior to applying the donor solution, the cells were incubated in a warm water bath. After 1 hour, each cell was checked for leakage and the presence of air bubbles. 250 µL of the donor solution was applied to each donor compartment. Receptor fluid samples were taken at regular time intervals and analyzed by HPLC-UV or by liquid scintillation counting for paromomycin sulphate. Flux and lag time were calculated using the linear portion of the graph when the cumulative amount of drug permeated was plotted as a function of time. Statistical analysis were performed using SPSS software version 19.0. The permeability coefficient was calculated according to the following equation:

$$J = K_p C_0$$

Whereby J is the flux of the permeant per unit area (in $mol \cdot cm^{-2} \cdot s^{-1}$) and C_0 is the concentration of drug applied to the skin surface (in $mol \cdot cm^{-3}$).

The saturated drug concentration was determined by adding an excess amount of drug to PBS in a clean vial. A magnetic stirrer was added and the suspension was left to stir for 48 hours at 32 ˚C. An aliquot of the

suspension was centrifuged at 13000 rpm at 32 ˚C for 30 minutes. The supernatant was taken and analysed by HPLC-UV.

Drug quantification

HPLC-UV. All compounds, except paromomycin, were analysed using a 1260 Infinity Agilent HPLC system. The column and settings used to separate and detect the compounds are summarised in Table 1.

Liquid scintillation counting. Because paromomycin sulfate lacks ultraviolet absorbing chromophore functions HPLC-UV could not be used to analyse the permeation samples. Therefore the donor solution was spiked with radiolabeled paromomycin sulfate and permeation samples were analysed using liquid scintillation counting. Thus 100 µL of receptor fluid and 100 µL of Optiphase™ supermix cocktail (Perkin Elmer, Buckinghamshire, UK) were added to a 96-well flexible PET microplate (Perkin Elmer, Buckinghamshire, UK). The plates were incubated on a plate shaker for 1 hour at room temperature after which they were fitted in an appropriate support cassette and placed in the MicroBeta® Trilux with two detectors for scintillation counting (1 minute/well).

Each plate included 3 blanks containing PBS that had been in contact with mouse skin (100 µL of PBS), 3 negative controls (100 µL of paromomycin sulfate saturated solution without radiolabeled compound) and 12 standards (1/5 dilutions). Additionally 3 samples of the donor solution were analysed to confirm homogeneous distribution of the radiolabeled compound in the solution. The counts per minute readings

obtained for the samples were converted to mg of paromomycin sulfate/ml using the standard curve.

Results

Histological changes in *Leishmania*-infected skin

A histological examination of the epidermal and dermal layers indicated structural skin changes upon infection with *Leishmania* parasites, as shown in Figures 1 and 2. The following changes to infected skin were observed: (i) an upward movement of the skin layers due to the infiltration of cells, with a disruption of the continuity of the epidermal and dermal skin layers at the centre of the lesion (Figure 1 (C)), (ii) an increased number of epidermal cell layers compared to uninfected skin (Figures 1 (B) and (D)) suggesting epidermal hyperplasia, (iii) abundant presence of macrophage-like cells in the dermal and lower epidermal layers (Figure 2 (B)), (iv) and collagen fibres were more spread out and less dense in infected skin (Figure 2 (D)), compared to the dense network of fibres in uninfected skin (Figure 2 (C)).

Transepidermal water loss (TEWL), skin hydration and pH of *Leishmania*-infected mouse skin

Changes in TEWL of infected and uninfected mouse skin with time are shown in Figure 3. Baseline TEWL values (prior to infection) in both groups were similar (t-test; $p>0.05$) indicating no difference in the 'inside-outside' skin barrier function between the groups. TEWL values increased slightly in both groups on day 2 potentially due to temporary

skin damage caused by the needle insertion. By day 10, when the infected mice had developed a nodule with a diameter of 5.4±0.7 mm, the mean TEWL values in the infected group had increased significantly compared to the uninfected group (t-test, $p<0.05$).

There were no significant differences in skin pH and hydration recorded over time (repeated measures ANOVA, $p >0.05$) with average values of pH 5.8±0.3 and 5.6%±0.6 hydration and pH 5.8±0.3 and 6.0%±0.5 hydration in infected and uninfected mice respectively.

Ex vivo permeability of *Leishmania*-infected skin

The cumulative amount of each test molecule in the receptor compartment of Franz diffusion cells, as a function of time, is shown in Figure 4 and permeation derived parameters such as lag time, flux and permeability coefficients are shown in Table 2.

Infected skin was more permeable to the model molecules compared to uninfected skin. Both caffeine and ibuprofen, applied as a saturated solutions, showed a higher permeation through infected skin compared to uninfected skin. At the end of the 48-hour experiment, the cumulative amount of caffeine that had permeated through infected skin was 2 to 47 times higher compared to uninfected skin (t-test, $p < 0.05$), the flux was 66 times higher and the permeability coefficient was 50 times higher for infected skin compared to uninfected skin ($p<0.05$). For ibuprofen, the corresponding differences between infected and uninfected skin were more modest, although they were still statistically significant. After 48 hours of permeation, the cumulative amount of permeated ibuprofen was 2 to 3 times higher while the flux and permeability coefficient were twice

as high for the infected skin compared to the uninfected skin (t-test, $p<0.05$). Interestingly, for both caffeine and ibuprofen, there was no difference in lag times for infected an uninfected skin (t-test, $p >0.05$).

For the anti-leishmanial drugs, uninfected skin was impermeable to the test compounds and no drugs could be detected in the receptor phase throughout the 48-hour permeation experiment. In contrast, the permeability of infected skin depended on the drug, being impermeable to amphotericin B, slightly permeable to buparvaquone, and highly permeable to paromomycin.

When the permeation profiles and parameters of all the tested compounds through infected skin are compared, paromomycin sulphate had the highest flux compared to caffeine, ibuprofen and buparvaquone (one-way ANOVA, $p<0.05$) while buparvaquone had the highest lag time and permeability coefficient compared to paromomycin, caffeine and ibuprofen ($p >0.05$ for both lag time and K_p).

Discussion

A number of approaches were used to characterise skin barrier integrity, an important parameter that influences topical drug delivery to *Leishmania*-infected skin. We first examined the histopathological changes in skin physiology. The most outstanding observation was the ingress of abundant inflammatory cells to the place of infection which disturbed the continuity of the epidermal and dermal skin layers. This is similar to previous reports of inflammation in BALB/c mice that are

highly susceptible to *Leishmania major* infection, and where lesions are characterised by extensive inflammation, ulceration and necrosis. [18-21] The disturbance to the epidermal and dermal skin layers, shown by the reduced density of the collagen and epidermal hyperplasia, indicates that the skin barrier properties may be reduced; abnormalities in the epidermal layer such as proliferation and differentiation being known to be essential for the integrity of the skin barrier function. [22] Indeed, reduced skin barrier properties were observed. TEWL – an indication of how well the skin controls water movement from the interior of the body – was doubled in *Leishmania*-infected skin. Such increase in TEWL was also observed in a number of other skin diseases such as tinea infections, psoriasis. [11, 23, 24]

Interestingly, the skin hydration, measured by the moisture sensor, did not change upon infection. This was surprising as inflammation in the infected lesion and the subsequent oedema was expected to increase the water content in the skin. Our results may be explained by the fact that the moisture sensor only measures the moisture content of the stratum corneum, [25] and not that of the whole skin. It appears that despite fluid accumulation in the *Leishmania* lesion, the surface of the skin does not become more hydrated compared to uninfected skin.

Uninfected mouse skin had an acidic surface, correlating with the “acid mantle” term used to describe the inherent acidic nature of the stratum corneum, [26] and the pH value of 5.8 is similar to previously reported value of 5.9 for hairless mouse skin. [27] Skin surface pH is known to be influenced by certain diseases, for example, tinea infection in humans

resulted in a higher foot skin pH. [28] The absence of any difference in the pH of *Leishmania*- infected and uninfected skin could be due to the fact that *Leishmania* infection was not located in the stratum corneum. Instead, as shown in Figure 2E, the *L. major* amastigotes were present in the dermis.

The reduced barrier function of *Leishmania*-infected skin indicated by TEWL (as discussed above) was confirmed in permeation experiments. However, the permeant's size and lipophilicity play a critical role in its permeation (or lack of). The hydrophilic molecules paromomycin sulphate and caffeine showed much larger increases in flux compared to the more lipophilic molecules ibuprofen and buparvaquone through infected skin. The presence of *Leishmania* parasites in the skin triggers a cascade of reactions resulting in inflammation and release of chemokines. [29, 30] It seems that the subsequent oedema, caused by additional fluid accumulating in the interstitial spaces, facilitates permeation of the more water soluble compounds such as caffeine and paromomycin sulphate while the permeation of drugs with low water solubility such as buparvaquone was not increased to the same extent.

Another possible reason for the enhanced permeation of the water soluble compounds could be the greater concentration gradient from donor to receptor phases for compounds with a higher aqueous solubility. All the permeants were applied as saturated solutions and therefore had a thermodynamic activity of 1. The rate of diffusion of molecules through a membrane is proportional to the concentration gradient. For the highly -water soluble paromomycin sulfate, this gradient is nearly 25 times

higher compared to caffeine and more than 4000 times higher compared to ibuprofen. This is likely to contribute to this increased permeation for more water soluble compounds.

In contrast to paromomycin, buparvaquone's flux through infected skin is hampered by its low water solubility and it's high lipophilicity, as suggested for healthy skin. [17] Low water solubility means low drug concentration in the donor phase and hence low concentration gradient across the skin, while drugs with a high lipophilicity means that the drug is able to quickly partition into the lipid component of the stratum corneum and remains there preferentially, such that a very long lag time and low flux through the damaged infected skin is observed.

As mentioned above, permeant size was also critical to its flux through infected skin. Amphotericin B with a molecular weight of 924 g/mol proved to be too large, as well as too water-insoluble, a molecule to permeate through uninfected or infected skin. This reflects previous *in vitro* [31, 32] and *in vivo* [33-35] studies and explains why previous studies in mice reported no cure of leishmaniasis lesions after topical application of amphotericin B (a potent anti-leishmanial drug), [36] despite its efficacy when administered through other routes, for example intradermally. [20]

In contrast to the mouse study by El-On et al (1984), one clinical study reported reduction in lesion size of cutaneous leishmaniasis lesions following the application of amphotericin B in solvents such as ethanol, [34] this was possibly due to skin lipid removal by ethanol leading to reduced barrier function and a potential increase in drug permeation. In

another clinical study on ulcerated lesions, the topical application of the lipidic formulation Amphocil in 5% ethanol Vardy et al. (2001) reported a faster healing rate compared to placebo except for one lesion. [37] In this case, the major reason for lesion healing caused by amphotericin B would be the absence of the stratum corneum in CL ulcers, i.e the absence of any barrier to the topically-applied drug from its site of action.

In addition, amphotericin B, paromomycin and buparvaquone were also unable to permeate through healthy uninfected skin. Paromomcyin also has a high molecular weight and physicochemical properties that are unfavourable for skin permeation. This was reflected in the lack of permeation through uninfected skin in our study, as described previously by Nogueira et al. (2009) who evaluated the permeation of different paromomycin salts through mouse skin. [38] In contrast the permeation of paromomycin through infected skin was expected as Aguiar et al. (2009) reported systemic absorption after topical application on ulcerated CL lesions in BALB/c mice[39]. It is important to note that the absence of the SC in such ulcerated lesions is likely to enhance the permeation into the dermis where the drug enters the blood circulation; this differs from the conditions in our experiment where the permeation through nodular CL lesions, with epidermis and SC, was evaluated.

The inter-individual variability in our permeation profiles (Figure 4) was high. Similar variability, has been reported in previous clinical studies [40-42] and was ascribed to differences in skin lipid composition and stratum corneum thickness between the test subjects. In this study inbred BALB/

mice were used to mitigate inter-individual variability. However, possible changes caused experimental manipulations as suggested by [43, 44] could not be avoided. Moreover, the permeation experiments which were conducted on infected skin, included variations in the progression of the infection in individual mice, despite the use of a standardised inoculum of *L. major* parasites to create lesions.

Despite the variability, we found a greater permeability of *Leishmania*-infected skin and the influence of the permeant's molecular mass and water-solubility/hydrophilicity was established. The importance of this data in relation to treatment of clinical CL is at present unclear due to known differences in mouse and human skin, including (i) the thinner mouse skin and mouse stratum corneum, [45] (ii) the absence of sweat glands in mouse skin, and (iii) the higher number of hair follicles in mouse skin and (iv) the different composition of intercellular stratum corneum lipids [46] which can all contribute to the higher permeability of mouse skin. [47-49] Further studies on human skin are required to determine whether these alterations in mouse skin are predictive of similar changes in human skin. This is important in relation to both the potential of new anti-leishmanial drugs in topical formulations, and an approach towards the direct administration of topical formulations on intact skin over CL nodules or around CL ulcers, where the infection is found in the dermis, which should avoid the need for removal of the ulcerative crust, washing and occlusion. [3]

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Transparency declarations

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References

1. Alvar J, Velez ID, Bern C et al. Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS One* 2012; **7**: e35671.
2. den Boer M, Argaw D, Jannin J et al. Leishmaniasis impact and treatment access. *Clin Microbiol Infect* 2011; **17**: 1471-7.
3. Ben Salah A, Ben Messaoud N, Guedri E et al. Topical paromomycin with or without gentamicin for cutaneous leishmaniasis. *N Engl J Med* 2013; **368**: 524-32.
4. Layegh P, Rajabi O, Jafari MR et al. Efficacy of Topical Liposomal Amphotericin B versus Intralesional Meglumine Antimoniate (Glucantime) in the Treatment of Cutaneous Leishmaniasis. *J Parasitol Res* 2011; **2011**: 656523.
5. Neal RA, Murphy AG, Olliaro P et al. Aminosidine ointments for the treatment of experimental cutaneous leishmaniasis. *Trans R Soc Trop Med Hyg* 1994; **88**: 223-5.
6. González U, Pinart M, Reveiz L et al. Interventions for Old World cutaneous leishmaniasis. *Cochrane Database Syst Rev* 2008: 111.
7. Aoyagi T, Terashima O, Suzuki N et al. Polymerization of benzalkonium chloride-type monomer and application to percutaneous drug absorption enhancer. *J Control Release* 1990; **13**: 63-71.
8. Williams AC, Barry BW. Penetration enhancers. *Adv Drug Deliv Rev* 2004; **56**: 603-18.
9. Moreno E, Schwartz J, Fernandez C et al. Nanoparticles as multifunctional devices for the topical treatment of cutaneous leishmaniasis. *Expert Opin Drug Deliv* 2014; **11**: 579-97.
10. Jakasa I, Verberk MM, Esposito M et al. Altered penetration of polyethylene glycols into uninvolved skin of atopic dermatitis patients. *J Invest Dermatol* 2007; **127**: 129-34.
11. Jensen JM, Pfeiffer S, Akaki T et al. Barrier function, epidermal differentiation, and human beta-defensin 2 expression in tinea corporis. *J Invest Dermatol* 2007; **127**: 1720-7.
12. Cork MJ, Danby SG, Vasilopoulos Y et al. Epidermal Barrier Dysfunction in Atopic Dermatitis. *J Invest Dermatol* 2009; **129**: 1892-908.
13. Anigbogu AN, Williams AC, Barry BW. Permeation characteristics of 8-methoxypsoralen through human skin; relevance to clinical treatment. *J Pharm Pharmacol* 1996; **48**: 357-66.
14. Gould AR, Sharp PJ, Smith DR et al. Increased permeability of psoriatic skin to the protein, plasminogen activator inhibitor 2. *Arch Dermatol Res* 2003; **295**: 249-54.
15. Akomeah FK, Martin GP, Brown MB. Variability in human skin permeability in vitro: comparing penetrants with different physicochemical properties. *J Pharm Sci* 2007; **96**: 824-34.
16. Ng SF, Rouse JJ, Sanderson FD et al. Validation of a static Franz diffusion cell system for in vitro permeation. *Aaps Pharmscitech* 2010; **11**: 1432-41.
17. Garnier T, Mantyla A, Jarvinen T et al. Topical buparvaquone formulations for the treatment of cutaneous leishmaniasis. *J Pharm Pharmacol* 2007; **59**: 41-9.
18. Tonkal A. Histopathology of the inflammatory response in experimental cutaneous leishmaniasis. *Department of infection and immunology* London School of Hygiene and Tropical Medicine, 1993.
19. Belkaid Y, Kamhawi S, Modi G et al. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* Infection in the mouse ear dermis. *J Exp Med* 1998; **188**: 1941-53.

20. Corware K, Harris D, Teo I et al. Accelerated healing of cutaneous leishmaniasis in non-healing BALB/c mice using water soluble amphotericin B-polymethacrylic acid. *Biomaterials* 2011; **32**: 8029-39.
21. Cangussu SD, Souza CC, Campos CF et al. Histopathology of Leishmania major infection: revisiting *L. major* histopathology. *Mem Inst Oswaldo Cruz* 2009; **104**: 918-22.
22. Ekanayake-Mudiyanselage S, Aschauer H, Schmook FP et al. Expression of epidermal keratins and the cornified envelope protein involucrin is influenced by permeability barrier disruption. *J Invest Dermatol* 1998; **111**: 517-23.
23. Motta S, Monti M, Sesana S et al. Abnormality of water barrier function in psoriasis. Role of ceramide fractions. *Arch Derm* 1994; **130**: 452-6.
24. Tomita Y, Akiyama M, Shimizu H. Stratum corneum hydration and flexibility are useful parameters to indicate clinical severity of congenital ichthyosis. *Exp Dermatol* 2005; **14**: 619-24.
25. Scalar 2015. Skin moisture sensor MY-808S. http://www.scalar.co.jp/english/detail.php?id=my-808s (Accessed 24/06/2015).
26. Schade H, Marchionini A. Der Säuremantel der Haut (nach Gaskettenmessung). *Klinische Wochenschrift* 1928; **7**: 12-4.
27. Turner NG, Cullander C, Guy RH. Determination of the pH gradient across the stratum corneum. *J Investig Dermatol Symp Proc* 1998; **3**: 110-3.
28. Chikakane K, Takahashi H. Measurement of skin pH and its significance in cutaneous diseases. *Clin Derm* 1995; **13**: 299-306.
29. Beil WJ, Meinardus-Hager G, Neugebauer DC et al. Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice. *J Leukoc Biol* 1992; **52**: 135-42.
30. Ritter U, Korner H. Divergent expression of inflammatory dermal chemokines in cutaneous leishmaniasis. *Parasite Immunol* 2002; **24**: 295-301.
31. Manosroi A, Kongkaneramit L, Manosroi J. Stability and transdermal absorption of topical amphotericin B liposome formulations. *Int J Pharm* 2004; **270**: 279-86.
32. Hussain A, Samad A, Nazish I et al. Nanocarrier-based topical drug delivery for an antifungal drug. *Drug Dev Ind Pharm* 2014; **40**: 527-41.
33. Yardley V, Croft SL. Activity of liposomal amphotericin B against experimental cutaneous leishmaniasis. *Antimicrob Agents Chemother* 1997; **41**: 752-6.
34. Frankenburg S, Glick D, Klaus S et al. Efficacious topical treatment for murine cutaneous leishmaniasis with ethanolic formulations of amphotericin B. *Antimicrob Agent Chemother* 1998; **42**: 3092-6.
35. Ruiz HK, Serrano DR, Dea-Ayuela MA et al. New amphotericin B-gamma cyclodextrin formulation for topical use with synergistic activity against diverse fungal species and Leishmania spp. *Int J Pharm* 2014.
36. El-On J, Jacobs GP, Witztum E et al. Development of topical treatment for cutaneous leishmaniasis caused by Leishmania major in experimental animals. *Antimicrob Agents Chemother* 1984; **26**: 745-51.
37. Vardy D, Barenholz Y, Naftoliev N et al. Efficacious topical treatment for human cutaneous leishmaniasis with ethanolic lipid amphotericin B. *Trans R Soc Trop Med Hyg* 2001; **95**: 184-6.
38. Nogueira IR, Carneiro G, Yoshida MI et al. Preparation, characterization, and topical delivery of paromomycin ion pairing. *Drug Development and Industrial Pharmacy* 2011; **37**: 1083-9.
39. Aguiar MG, Silva DL, Nunan FA et al. Combined topical paromomycin and oral miltefosine treatment of mice experimentally infected with Leishmania (Leishmania) major leads to reduction in both lesion size and systemic parasite burdens. *Journal of Antimicrobial Chemotherapy* 2009; **64**: 1234-40.

40. Larsen RH, Nielsen F, Sorensen JA et al. Dermal penetration of fentanyl: inter- and intraindividual variations. *Pharmacol Toxicol* 2003; **93**: 244-8.
41. Scott RC, Corrigan MA, Smith F et al. The influence of skin structure on permeability: an intersite and interspecies comparison with hydrophilic penetrants. *J Invest Dermatol* 1991; **96**: 921-5.
42. Schaefer UF, Hansen J, Schneider M et al. Models for skin absorption and skin toxicity testing. In: Ehrhardt C, Kim KJ, eds. *Drug absorption studies: in skin, in vitro, and in silico models*. New York: Springer Science, 2008; 3-33.
43. Southwell D, Barry BW, Woodford R. Variations in permeability of human skin within and between specimens. *Int J Pharm* 1984; **18**: 299-309.
44. Liu P, Nightingale JAS, Kurihara-Bergstrom T. Variation of human skin permeation in vitro: Ionic vs neutral compounds. *Int J Pharm* 1993; **90**: 171-6.
45. Wester RC, Maibach HI. *In vivo* methods for percutaneous absorption measurements. In: Brounaugh RL, Maibach HI, eds. *Percutaneous absorption: mechanisms-methodology-drug delivery*. New York: Marcel Dekker Inc., 1989; 215–37.
46. Netzlaff F, Schaefer UF, Lehr CM et al. Comparison of bovine udder skin with human and porcine skin in percutaneous permeation experiments. *Altern Lab Anim* 2006; **34**: 499-513.
47. Harada K, Murakami T, Kawasaki E et al. In-vitro permeability to salicylic acid of human, rodent, and shed snake skin. *J Pharm Pharmacol* 1993; **45**: 414-8.
48. Roy SD, Hou SY, Witham SL et al. Transdermal delivery of narcotic analgesics: comparative metabolism and permeability of human cadaver skin and hairless mouse skin. *J Pharm Sci* 1994; **83**: 1723-8.
49. Roberts ME, Mueller KR. Comparisons of in vitro nitroglycerin (TNG) flux across yucatan pig, hairless mouse, and human skins. *Pharm Res* 1990; **7**: 673-6.
50. Gaspari F, Bonati M. Correlation between n-octanol/water partition coefficient and liquid chromatographic retention for caffeine and its metabolites, and some structure-pharmacokinetic considerations. *J Pharm Pharmacol* 1987; **39**: 252-60.
51. Herkenne C, Naik A, Kalia YN et al. Ibuprofen transport into and through skin from topical formulations: in vitro-in vivo comparison. *J Invest Dermatol* 2007; **127**: 135-42.
52. Mantyla A, Garnier T, Rautio J et al. Synthesis, in vitro evaluation, and antileishmanial activity of water-soluble prodrugs of buparvaquone. *J Med Chem* 2004; **47**: 188-95.

Table 1. Details of the HPLC methods for caffeine, ibuprofen, amphotericin B and buparvaquone.

Compound ID	HPLC column	Injection volume (µl)	Mobile phase		Flow rate (ml/min)	Detector wavelength (nm)
			A	B		
Caffeine	Phenomenex; Synergi–Hydro RP (250x4.6mm; 5µm)	20	0.1% TFA in water (15%)	ACN (85%)	1.3	273
Ibuprofen	Phenomenex; Synergi–Hydro RP (250x4.6mm; 5µm)	20	0.1% TFA in water (30%)	ACN (70%)	1	227
Amphotericin B	Phenomenex; Synergi–Hydro RP (250x4.6mm; 5µm)	80	2.5mM EDTA in water (68%)	ACN (37%)	1	407
Buparvaquone	Luna C18 (4.6x250mm; 5µm) with Luna guard column	20	5% Water+ acetic acid (pH3.5) (5%)	MeOH (95%)	1.7	250

Table 2. The physico-chemical properties (solubility, log D and molecular weight) and permeation parameters (flux, lag time and permeability coefficient (K_p)) for the drugs tested (mean+/-SD, n=5).

	caffeine	ibuprofen	paromomycin sulphate	amphotericin B	buparvaquone
Solubility (mg/ml)					
PBS (pH 7.4)	22.74±5.26	0.15±0.01	>650000	< 0.75	<0.1
PBS+2%CD			N/A	37±4	853±25
Log D	-0.07 (pH 3.0)[1]	4.0[2]	-2.9[3]	-0.66[3]	7.0 (pH 3.0)[4]
Mol. weight (g/mol)	194	206	714	924	326
Flux (µg/cm^2/h)					
uninfected	0.6±0.1	7.3±1.2	Not detected	Not detected	Not detected
infected	40.1±43.8	15.0±3.7	548±201	Not detected	0.17±0.1
Lag time (h)					
uninfected	14.9±5.2	11.4±1.1	Not detected	Not detected	Not detected
infected	12.7±3.5	10.7±0.5	15.4±4.0	Not detected	25.32±3.1
K_p (cm/h)					
uninfected	2.3E-05±5.5E-06	0.05±0.01	Not detected	Not detected	Not detected
infected	1.1E-03±8.5E-04	0.11±0.03	7.01E-04	Not detected	1.75±1.0

[1] [50]

[2] [51]

[3] (estimated using ChemBioDraw Ultra 13.0)

[4] [52]

Figure 1. Histology sections of BALB/c mouse skin (H&E stain) showing the *L. major* skin on the rump in an infected and uninfected biopsy. (A and B) uninfected skin at day 11 after injection of medium without parasites, (B) is a magnification of the epidermis in uninfected skin indicated by the bracket; (C, D and E) infected skin at day 11 after infection, (D) is a magnification of the epidermis in infected skin indicated by the bracket (E) is a magnification of the lower dermal layer of infected skin and shows the presence of *L. major* amastigotes in the phagolysosomes of macrophages indicated by the circles.

Figure 2. Histology sections of BALB/c mouse skin showing the *L. major* skin on the rump in an infected and uninfected biopsy. (A) Iba-1 stain of uninfected skin at day 11 after injection of medium without parasites, (B) Iba-1 stain of infected skin at day 11 after infection. The abundant brown staining indicates infiltration of numerous macrophage-like cells in the dermis. (C) Von Gieson staining of uninfected skin on day 11. The collagen fibres present in the dermis are visualized by an intense red stain, (D) Von Gieson staining of infected skin on day 11 after infection. The staining is less intense and the fibres are spaced by invading inflammatory cells. Moreover the thickening of the epidermis, the outer layer of the skin, is clearly visible.

Figure 3. TEWL and lesion size evolution in time in infected and uninfected skin (average±SD). TEWL values are significantly higher for the *L. major* infected skin (n=10) compared to the uninfected skin (n=6) on day 10 after infection, when a nodule size of 5-6mm diameter was reached (repeated-measures ANOVA, $p < 0.05$; n=5 in each group). This indicates an impaired in to outside skin barrier.

Figure 4.The cumulative amount of caffeine, ibuprofen, buparvaquone and paromomycin sulphate permeated per surface area (µg/cm^2) through uninfected and infected skin as a function of time (h). The permeation profiles are of individual skin samples. Each mouse provided 2 skin samples, one infected (filled symbol) and one uninfected (unfilled symbol).