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1 **Title:** Topical treatment for cutaneous leishmaniasis – dermato-pharmacokinetic led
2 optimisation of benzoxaboroles

3

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5

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19

20 **Abstract**

21 Cutaneous leishmaniasis (CL) is caused by several species of the protozoan parasite *Leishmania*
22 – affecting an estimated 10 million people worldwide. Previously reported strategies for the
23 development of topical CL treatments have focussed primarily on drug permeation and
24 formulation optimisation as the means to increase treatment efficacy.

25 Our approach aims to identify compounds with anti-leishmanial activity and properties
26 consistent with topical administration. Of the test compounds, five benzoxaboroles showed
27 potent activity ($EC_{50} < 5 \mu\text{M}$) against intracellular amastigotes of at least one *Leishmania* species
28 and acceptable activity ($20 \mu\text{M} < EC_{50} < 30 \mu\text{M}$) against two more species. Benzoxaborole
29 compounds were further prioritised based upon the *in vitro* evaluation of progression criteria
30 related to skin permeation such as the partition coefficient and solubility. An MDCK-MDR1
31 assay showed overall good permeability and no significant interaction with the P-glycoprotein
32 transporter for all substrates except LSH002 and LSH031. The benzoxaboroles were degraded,
33 to some extent, by skin enzymes but have superior stability than para-hydroxybenzoate
34 compounds that are known skin esterase substrates. Permeation evaluation through
35 reconstructed human epidermis showed LSH002 to be most permeable followed by LSH003 and
36 LSH001. Skin disposition studies following finite drug formulation application to mouse skin
37 demonstrated the highest permeation for LSH001 followed by LSH003 and LSH002 with a
38 significantly higher amount of LSH001 retained in skin compared to other compounds.

39 Finally, the efficacy of the leads (LSH001, LSH002 and LSH003) was tested *in vivo* against
40 *Leishmania major*. LSH001 suppressed lesion growth upon topical application and LSH003

41 reduced the lesion size following oral administration.

42

43 Introduction

44 The leishmaniasis are a group of neglected tropical diseases, caused by the obligate
45 intracellular protozoan parasite *Leishmania* that mainly occurs in low- to middle-income
46 countries. Leishmaniasis is endemic in 98 countries over five continents placing 350 million
47 people at risk of infection (1). Over 17 different *Leishmania* species can cause a variety of
48 clinical symptoms that depend both on host and parasite related factors.

49 The most common form CL is widely distributed, with 70-75% of the estimated cases occurring
50 in Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru
51 (1) and continues to spread due to environmental changes such as deforestation, travel,
52 emigration and agricultural practice (2-5). In its simplest form, CL presents as a single local skin
53 lesion that tends to heal spontaneously over a period of 3-18 months leaving scars (6).
54 However, a range of clinical manifestations of variable severity are observed in patients that do
55 not achieve spontaneous clearance of the parasite. These manifestations include nodules,
56 ulcers and plaques depending upon the *Leishmania* species causing the infection and the status
57 of host immune system (7). Immediate treatment is vital to expedite healing, reduce scar
58 formation, prevent relapse or to prevent parasite dissemination.

59 Drugs commonly utilised to treat CL such as pentavalent antimonials, miltefosine, amphotericin
60 B and paromomycin are limited by parenteral drug administration, toxicity, variable efficacy
61 and cost. Over the past decade, despite efforts in screening and drug discovery to identify new
62 chemical series for visceral leishmaniasis (8, 9), only a few novel chemical classes have been
63 explored for CL. Instead, research mainly focussed on repurposing existing drugs or novel

64 formulation strategies. For example amphotericin B, currently approved for parenteral delivery
65 has been evaluated for topical delivery in formulations (10) including lipid nano-carriers (11,
66 12), nano-emulsions (13) or cyclodextrin complexes (14). Similarly, the anti-leishmanial drug
67 paromomycin was formulated in conventional topical vehicles (15-18) and in novel delivery
68 systems including liposomes (19) in an attempt to increase skin permeation. However, the
69 physicochemical properties of both drugs are unfavourable for skin permeation and the
70 reformulation strategies for these compounds have met with limited success.

71 To enable the further development of treatments for CL we previously characterised how
72 *Leishmania* infection impacts the permeability of the skin barrier and how this might influence
73 topical drug delivery during the acute phase of the treatment (20). These studies have
74 demonstrated that the skin barrier is compromised during the nodular stage of CL suggesting a
75 weaker barrier to dermal delivery.

76 Besides identifying disease related changes to drug delivery, the identification of drug
77 compounds that are active against a broad range of *Leishmania* parasites is also key (21).
78 Benzoxaborole compounds, characterised by the boron atom incorporated in a ring system
79 fused to an aromatic ring (Table 1), have previously shown activity against bacteria, fungi and
80 protozoans such as *Trypanosoma brucei* and *Plasmodium falciparum* (22-27). Phenotypic
81 screenings of a library of benzoxaboroles identified *in vitro* and *in vivo* activity of
82 benzoxaboroles 6-carboxamides against *T. brucei* and *T. cruzi*, the causative agents of human
83 African trypanosomiasis (HAT) and Chagas disease (22, 28), respectively. Additionally, more
84 than 2000 compounds were evaluated against *L. donovani* amastigotes in THP-1 cells to identify

85 drugs to treat visceral leishmaniasis and resulted in several hits with micromolar activity (DNDi
86 funded work, unpublished data).

87 Here we describe an approach for the rational pre-clinical selection of candidate molecules for
88 CL (Figure 1 (A)), using a series of benzoxaboroles that were found to (i) demonstrate activity
89 against a selection of *Leishmania* species (ii) have the ability to permeate skin and (iii) were
90 appropriately distributed in various skin layers (Figure 1 (B)).

91

92 **Materials and Methods**

93 **Materials**

94 Compounds were synthesised by Anacor Pharmaceuticals Inc. and SCYNEXIS Inc. (Research
95 Triangle Park, NC) and were of >95% purity as determined by HPLC, LC-MS and ¹H-NMR
96 analyses. Stock solutions (1 mM) were prepared in dimethyl sulfoxide (DMSO) for use in the *in*
97 *vitro* experiments. HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA).
98 Formic acid (≥98% purity, Fluka), caffeine, testosterone, 1-octanol, high grade vacuum silicone
99 grease (Dow Corning) were acquired from Sigma-Aldrich (St. Louis, MO). Ammonium formate
100 (99% purity, Alpha Aesar) was purchased from VWR International, LLC (West Chester, PA).
101 Miglyol 840 (propylene glycol dicaprylate / dicaprinate) was obtained from Sasol Germany GmbH
102 (Witten, Germany). Phosphate buffered saline (PBS) was supplied by Gibco (Invitrogen
103 Corporation, Carlsbad, CA) as well as the Dulbecco's modification of Eagle's medium with
104 GlutaMAX, the trypsin-EDTA and the Fetal Bovine Serum. Penicillin-Streptomycin solution,
105 Hank's balanced salt solution and HEPES buffer were obtained from Sigma Aldrich.

106 **Mice**

107 Female BALB/c mice (6-8 weeks old) were purchased from Charles River (Margate, UK) and
108 housed in a controlled environment of 55% relative humidity and 26°C. Tap water and a
109 standard laboratory diet were provided ad libitum. All *in vivo* experiments were carried out
110 under license (PPL 70/8207) at the London School of Hygiene & Tropical Medicine (LSHTM)
111 after discussion with the veterinarian, clearance through the LSHTM Animal Welfare and Ethical
112 Review Board and according to UK Home Office regulations.

113 **Parasite and cell maintenance**

114 *L. major* (MHOM/SA/85/JISH118); *L. panamensis* (MHOM/PA/67/BOYNTON); *L. aethiopica*
115 (MHOM/ET/84/KH); *L. mexicana* (MNYC/ BZ/62/M379) and *L. tropica* (MHOM/IR/2013/HTD4)
116 were routinely passaged through BALB/c mice, and low passage number promastigotes
117 (typically below passage number 3) were used for the assays. All promastigotes, except for *L.*
118 *panamensis* and *L. aethiopica*, were maintained in Schneider's insect medium (Sigma Aldrich,
119 UK) supplemented with 10% heat inactivated foetal calf serum (HiFCS) (Harlan, UK) at 26°C.
120 M199 medium supplemented with 10% HiFCS was used for the latter two strains.

121 MDCKII-hMDR1 cells (Netherlands Cancer Institute, Amsterdam, Netherlands) were maintained
122 in Dulbecco's Modified Eagles Medium (DMEM) and KB cells in RPMI-1640 medium
123 supplemented with L-glutamine and 10% HiFCS. Both human-derived cell lines were left in an
124 incubator at 37°C and 5% CO₂ and passaged to new medium once a week (1/10 ratio).

125 ***In vitro* anti-leishmanial activity**

126 Mouse peritoneal macrophages (PEM) were isolated from CD-1 mice (Charles River, Margate,
127 UK) by abdominal lavage with RPMI-1640 medium containing 1% penicillin and streptomycin.
128 The collected cells were washed, re-suspended and seeded in 16-well Lab-Tek™ slide in RPMI-
129 1640 supplemented with 10% HiFCS at a density of 4x10⁴ per well. After 24 hours incubation at
130 37°C and 5% CO₂/95% air mixture, the adhered PEMs were infected with stationary phase
131 promastigotes at a ratio of 3 (for *L. tropica* and *L. major*) or 5 (for *L. mexicana*, *L. aethiopica* and
132 *L. panamensis*) promastigotes to 1 macrophage and maintained at 34°C in a 5% CO₂/95% air

133 mixture. These inoculum ratios were chosen to achieve at least 75% infection of untreated
134 control macrophages after 72 hours of incubation.

135 After 24 hours, the cultures were washed to remove extracellular promastigotes and one slide
136 was fixed with methanol and stained with Giemsa to determine the initial level of infection. If a
137 sufficient level of infection was obtained, experimental drug solutions over a range of 30, 10, 3
138 and 1 μ M were added in quadruplicate at each concentration. Amphotericin B (Fungizone[®]) and
139 miltefosine were included as control drugs. After 72 hours incubation, all slides were methanol-
140 fixed and Giemsa-stained.

141 The percentage inhibition was determined by microscopically (400x magnification) counting the
142 infected macrophages in drug treated cultures compared to untreated cultures. The Hill
143 coefficient, EC₅₀ and EC₉₀ values were calculated by non-linear sigmoidal curve fitting (variable
144 slope) using Prism Software (GraphPad, Surrey, UK).

145 ***In vitro* ADME studies—general pharmacokinetic predictions**

146 The following descriptors of the test compounds: molecular weight, aqueous solubility and
147 number of H-bond donors and acceptors present were calculated using ChemBioDraw Ultra
148 13.0 (PerkinElmer, Waltham, MA).

149 *Distribution coefficient.* The octanol phase was left to saturate with PBS (pH7.4) on a shaking
150 plate at 32°C for 48 hours. The test compounds were then dissolved in the 1-octanol at a
151 concentration of 1 μ g/ml and left to equilibrate with an equal volume of PBS on a shaking plate
152 at 32°C for 48 hours. The 1 μ g/ml concentration was selected such that the amount of the

153 candidate drug in each phase did not exceed 10% of the solubility limit of that compound.
154 Aliquots of each phase were taken and diluted in mobile phase followed by LC-MS/MS analysis.
155 Each experiment was conducted in triplicate. The distribution coefficient was calculated as
156 shown in Equation 1:

$$157 \quad \log D (\text{pH } 7.4) = \log \left[\frac{[\text{solute}]_{\text{oct}}}{[\text{solute}]_{\text{pbs}}^{\text{ion}} + [\text{solute}]_{\text{pbs}}^{\text{neutral}}} \right] \quad \text{Equation 1}$$

158 *In vitro prediction of permeability and Pgp-mediated efflux transport.* MDCK-MDR1 cells were
159 seeded in the apical chamber of a 12-well Transwell® plate (Corning Inc., Lowell, MA) at a
160 density of 6.6×10^6 cells/well and 1.5 mL of medium was applied in the basolateral chamber.
161 After 24 hours, non-adhered cells were washed away and new medium was applied to both
162 chambers. The cells were incubated for an additional 48 hours at 37°C to form confluent
163 monolayers.
164 Prior to the addition of the test compounds, the cell culture medium was removed and
165 replaced with transport medium consisting of Hanks's balanced salt solution with 24 mM of
166 glucose and 24 mM of HEPES buffer. The integrity of the monolayers was assured by measuring
167 the trans-epithelial resistance (TEER) for each insert (TEER > 160 Ω cm²). Assays were
168 performed in triplicate by adding 3 μ M drug solutions (1 mM DMSO stock solutions diluted in
169 transport medium) in the absence or presence of 2 μ M GF918 (a potent Pgp inhibitor (29)) in
170 the transport buffer of the apical chamber. The comparator controls propranolol and
171 amprenavir for transcellular transport and Pgp efflux respectively were included in each assay.
172 The Transwell® plates were incubated on a shaking plate (160 rpm) at 37°C and 5% CO₂ for 1
173 hour. After incubation, aliquots from both chambers were removed for analysis by LC-MS/MS.

174 Values for mass balance, apparent permeability for the apical to the basolateral side (P_{app})
175 (Equation 2), apparent permeability value for the apical to the basolateral in presence of
176 GF+918 ($P_{app+GF918}$), and the absorption quotient (AQ) (Equation 3) were calculated for each
177 compound (30-32). Test compounds with an $AQ \leq 0.3$ were considered non-Pgp substrates,
178 while $AQ > 0.3$ were considered Pgp substrates (31, 32). Acceptance criterion for mass balance
179 was 70–120%.

$$180 \quad P_{app} = \frac{dQ/dt}{C_0 \times A} \quad \text{EQUATION 2}$$

$$181 \quad AQ = \frac{P_{app+GF918} - P_{app}}{P_{app+GF918}} \quad \text{EQUATION 3}$$

182

183 Analysis of test compounds in biological samples

184 *Skin tissue homogenisation.* For the preparation of the skin homogenates, 20 ml of ice-cold
185 Dulbecco's modified PBS (pH 7.4) was added to fine pieces of approximately 2 g of shaved
186 dorsal full-thickness BALB/c mouse skin (Bioreclamation LLC., Westbury, NY). The tissue
187 suspension was homogenized using an OMNI probe homogenizer (Kennesaw, GA) and
188 centrifuged for 10 minutes at 800g to sediment cellular residue. The protein content of the
189 supernatant was determined using the Pierce BCA protein assay kit (Pierce, Rockford, IL) and
190 adjusted to 2.5 mg/ml. The supernatant was stored at approximately -80°C until use.

191 *High performance liquid chromatography with tandem mass spectrometry (LC-MS/MS).* Sample
192 analysis was performed by LC-MS/MS. The instrumentation consisted of a CTC Pal Autosampler
193 (Leap Technologies, Carrboro, NC), two Agilent 1100 series pumps (Agilent Technologies Inc.,

194 Santa Clara, CA), a CH-30 column heater (Eppendorf, Hauppauge, NY) and an API-3000 triple
195 quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a turbo-ion
196 electrospray interface for detection. Chromatography was performed on a Luna C18 reversed-
197 phase column (50 x 2 mm; 3 μ m) from Phenomenex (Torrance, CA) protected by a matched
198 phase guard column. The mass spectrometer and peripheral devices were controlled using
199 Analyst[®] Software version 1.4.2 (Applied Biosystems, Foster City, CA). The mobile phase used to
200 elute the compounds consisted of 5 mM ammonium formate and 0.1% (v/v) formic acid in
201 water (A) and 5 mM ammonium formate and 0.1% (v/v) formic acid in methanol (B). The
202 samples were introduced on the column using 90% A at a flow rate of 600 followed by a step
203 gradient to 90% B between 0.5 and 1min. For analytical chromatography, a linear gradient of
204 10% A was maintained for 2min after which the mobile phase was switched back to 90% A. This
205 mobile phase composition was maintained till the end of the run (3.5min). Test compounds
206 eluted between 2-3 min.

207 ***In vitro* stability and disposition in skin homogenates**

208 *Stability in skin homogenates.* The stability of the compounds was measured at protein
209 concentrations of 2.5 mg/ml. Each compound (10 μ M) was incubated in mouse skin
210 homogenate on a shaking plate at 32°C. An aliquot of the incubation mixture was collected at 0,
211 10, 20, 30 minutes, 1 hour and 2 hours and quenched with 4 volumes of ice-cold methanol
212 containing 0.1% formic acid. Samples were centrifuged at 3000xg for 10 minutes at 15°C, and
213 the obtained supernatant was analysed for the test compound by LC-MS/MS. Ethyl- and

214 propylparaben, ester compounds known to undergo degradation due to enzymatic hydrolysis
215 to yield hydroxybenzoic acid were included as positive controls.

216 *Skin tissue binding.* Rapid equilibrium dialysis (RED) devices (Pierce, Rockford, IL) in plate format
217 were used to determine the drug binding to the skin homogenate supernatant. A day prior to
218 the experiment, the Teflon plate was washed with 30% ethanol and rinsed twice with deionized
219 water before leaving it to dry. On the day of the experiment, skin supernatant was thawed and
220 the test compound was added to a final concentration of 10 μ M. Samples of fortified skin tissue
221 homogenate (300 μ l) were added to the sample chambers of the RED devices and PBS (500 μ l)
222 (Pierce, Rockford, IL) was added to each buffer chamber. Plates were incubated on a shaking
223 plate at 32°C for 2 hours. Aliquots of both phases were collected and treated with 4 volumes of
224 ice-cold methanol with 0.1% of formic acid to precipitate proteins. Treated sample aliquots
225 were centrifuged at 3000xg and 15°C for 10 minutes. The resulting supernatants were assayed
226 for the parent drug concentration by LC-MS/MS.

227 *In vitro prediction of skin permeability.* The EpiDerm™ Skin Model EPI-606-X was obtained from
228 MatTek Corporation (Ashland, MA, USA). The EPI-606-X model is characterised by an enhanced
229 barrier function and was specifically designed to conduct permeability assays. Upon receipt, the
230 skin tissue (lot 17860) was stored overnight at 2-8°C. On the day of the experiment, the skin
231 inserts were transferred to a 6-well plate containing 2ml of Dulbecco's modified PBS and left to
232 acclimatise on a heated shaking plate. The temperature was set at 36.6°C which corresponded
233 to a skin temperature of 32°C.

234 Due to a low water solubility, the test compounds were prepared in an ethanol/ Miglyol 840
235 (1:9) vehicle - a solution that has been used for permeation studies with poorly soluble drugs
236 (33). After 1 hour, 1.14 ml of a 100 µg/ml donor solution was applied on the model skin using a
237 positive displacement pipette. The plates were left to incubate with gentle shaking at 95 rpm.
238 Caffeine (log P=-0.08) and testosterone (log P=3.32) were included as control comparator
239 compounds in each assay run. Each control was evaluated at the same concentration as the test
240 compounds. Testosterone, representing a hydrophobic control, was formulated in the
241 ethanol/Myglyol vehicle, and caffeine representing a hydrophilic control, was prepared in
242 Dulbecco's modified PBS. Aliquots were removed from the receiver fluid of each chamber and
243 replaced with fresh PBS at regular time points over the course of 6 hours incubation. The
244 samples were assayed for test compound by LC-MS/MS. The permeation of each compound
245 was evaluated in triplicate. Statistical analyses were performed using SPSS software version
246 19.0.

247 **Skin disposition**

248 *In vitro permeation prediction using full-thickness BALB/c mouse.* *In vitro* permeation studies
249 were conducted in a semi-automated system comprising 6 water-jacketed, static, vertical type
250 Franz diffusion cells (FDC) from Logan instruments Ltd. (Somerset, NJ). The permeation studies
251 had two objectives (Table 2). The first objective was to compare the permeation of the test
252 compounds through BALB/c mouse skin to the permeability determined by means of the RHE
253 assay. Therefore, the experimental conditions were held consistent to those employed for the
254 RHE assay. The second objective was to compare the permeation of the test compounds using

255 the formulation conditions that would be used for topical dose administration in the murine
256 model of CL. This *in vivo* study required a low application volume and a 1% (w/v) drug
257 formulation.

258 For FDC studies, female BALB/c mouse skin was obtained from Bioreclamation IVT (Westbury,
259 NY, USA) and stored at -80°C. On the day of each study skin was thawed and hair removed by
260 careful clipping to avoid skin damage. Excess fat and muscle tissue was removed with the aid of
261 a scalpel. Discs of skin approximately 2.5cm in diameter were cut and mounted between the
262 donor and receptor compartment of each FDC and kept in place by the use of a clamp. Vacuum
263 silicone grease was applied to seal gaps and prevent leakage. The cells were left to equilibrate
264 until the skin temperature stabilised at 32°C.

265 The donor and receptor solutions were prepared as described above. Receptor fluid samples
266 were taken at time intervals over a period of 6 hours. Each test compound was tested in
267 triplicate. Statistical analysis was performed using SPSS software version 19.0.

268 *Mass balance during FDC studies.* Mass balance study was conducted using the formulations
269 and experimental conditions intended for evaluation in the murine model of CL. The amount of
270 drug that did not permeate into or through the skin (unabsorbed donor fraction) was obtained
271 by gently swabbing the skin surface with a cotton bud at the end of the permeation
272 experiment. This was repeated a second time. The cotton buds were placed in a tube with 1ml
273 of MeOH/PBS (70:30) and left overnight on a shaker (800 rpm). An aliquot of the extraction
274 fluid was analysed by LC-MS/MS.

275 The Franz diffusion cells were dismantled and the mouse skin was removed and placed in a vial.
276 Three rounds of extraction with 1ml of MeOH-PBS (7:3) were conducted. At each time, the vial
277 was left to shake overnight before analysis by LC-MS/MS to extract the amount of drug that
278 permeated into the skin. Acceptable mass balance was 80-120% representing the total
279 compound measured in the unabsorbed donor fraction, methanolic skin extracts, and the
280 samples of receptor chamber fluid. Statistical analyses were performed using SPSS software
281 version 19.0.

282 **Efficacy in a murine model of cutaneous leishmaniasis**

283 *Drugs and formulation preparation.* AmBisome[®], a liposomal formulation of amphotericin B for
284 injection was kindly provided by DNDi (Geneva) and prepared according to manufacturer's
285 recommendations. Briefly, AmBisome[®] powder was reconstituted with 12ml of cold sterile
286 ultra-high purity grade water (>18 MΩ.cm, MilliQ, Hertfordshire) to produce a 4 mg/ml
287 amphotericin B liposomal suspension. This suspension was vigorously shaken and incubated at
288 65°C for 10 minutes after which it was allowed to cool to room temperature. This dispersion
289 was diluted with sterile 5% dextrose solution (w/v) to obtain a final suspension of 0.5mg of
290 amphotericin B/ml. Every other day up to 5 doses, 200ul of this formulation was administered
291 by bolus intravenous injection into a lateral tail vein. Leshcutan[®] ointment, containing 12% of
292 paromomycin and 15% methylbenzethoniumchloride (Teva, Israel) was purchased from
293 Israelpharm.com and 0.1ml of a 1ml syringe was applied and gently spread over the nodule
294 twice daily for 10 days.

295 The experimental topical formulations containing compounds LSH001, LSH002 and LSH003
296 respectively were prepared 24 hours prior to the start of dosing. To allow maximal permeation,
297 each test compound was applied as a saturated solution. An excess amount of the test
298 compound was added to a 1:1 (v/v) mixture of propylene glycol (PG) and ethanol (Ethanol). The
299 mixture was left to stir overnight after which it was centrifuged at 15,668 x g for 15 minutes.
300 The supernatant, i.e. a saturated solution, was pipetted into a clean vial and 50ul was applied to
301 each mouse twice a day for 10 days (Table 3).

302 The standard suspension vehicle used to prepare the oral formulations was prepared by
303 weighing and adding each component (0.5% (w/v) carboxymethylcellulose, 0.5% (v/v) benzyl
304 alcohol, 0.4% Tween 80 (v/v) in a 0.9% (v/v) NaCl solution) into a clean glass vial. The mixture
305 was left to stir overnight at room temperature prior to sterilisation by autoclaving. The
306 experimental oral formulations containing either LSH001, LSH002 or LSH003 in the vehicle were
307 prepared by adding the appropriate amount of test compound to the vehicle in order to obtain
308 a final concentration of 2.5mg/ml. The suspension was sonicated for 30 minutes and was
309 administered orally twice a day for 10 days. All formulations, including the AmBisome and
310 topical formulations were stored at 4°C throughout the experiment.

311 *Experimental CL model.* Sixty female BALB/c mice (6-8 weeks old; Charles River Ltd., UK) were
312 shaved on the rump above the tail and one day later, injected with 2×10^7 stationary phase *L.*
313 *major* JISH118 promastigotes (200 μ l) subcutaneously on the rump above the tail.
314 Approximately 7 days post infection, small nodules were visible. The nodule size was recorded
315 daily and when they reached an average diameter of 4.8 mm (± 0.8), the mice were randomly

316 allocated in groups of 6 and drug administration was started. Formulations were administered
317 over a period of 10 days. Untreated and topical vehicle only control groups were included.

318 Treatment efficacy was evaluated by lesion size progression, measuring the lesion diameter in 2
319 dimensions on a daily basis using digital callipers (Jencons Scientific Ltd., UK). The average
320 diameter was plotted as a function of time. Statistical analyses of differences between the
321 average lesion diameter between groups on the last day of treatment was performed using
322 one-way ANOVA with post-hoc Tukey test (SPSS software version 19.0). Three days after the
323 end of treatment, the mice were sacrificed and the lesion was excised and stored at -80°C until
324 the parasite load was quantified using real-time qPCR. Statistical differences in the average
325 parasite numbers between different groups were analysed using one-way ANOVA with Tukey
326 post-hoc test (SPSS software, version 19.0).

327 *Quantification of the parasite load in a CL lesion.* On the day of extraction the samples of lesion
328 tissue were defrosted and cut into 2 approximately equal samples. One half was weighed and
329 cut into fine pieces with a surgical blade before placing in a microcentrifuge tube. The
330 proteinase K and lysis buffer were added to the tube and samples were incubated at 56°C until
331 a homogeneous mixture was obtained. The DNA of 200ul of this homogenate was then
332 extracted using the DNeasy[®] blood and tissue kit (Qiagen) and eluted in the same volume. The
333 purity and concentration of DNA was analysed using the NanoDrop™ ND1000
334 spectrophotometer (Thermo Fisher Scientific).

335 The primer pair and probe, previously designed and validated by Van Der Meide et al (34)
336 targeted a 170-bp region in the *Leishmania* 18S ribosomal gene and are specific for all

337 *Leishmania* species. The respective sequences are shown in Table 4. Conventional PCR was
338 performed to confirm the presence of PCR product of the correct size and to verify primer
339 efficacy. 1µl of a 1/100 dilution of the DNA extract was amplified in a final volume of 10µl
340 containing 2µl of KAPA 2G buffer (Kapa Biosystems, Wilmington, MA) and primers at a
341 concentration of 0.4µM. The samples were run in a G-Storm GS4 machine (Somerset, UK). The
342 amplification cycle started with a denaturation step at 95°C for 3 minutes followed by 40 cycles
343 of 95°C for 15s, 60°C for 1 minute and 72°C for 30 seconds with a final extension of 72°C for 30
344 seconds. Each run contained a negative sample whereby the extracted DNA was replaced by
345 UHP water. The PCR products were separated on a 3% agarose gel stained with ethidium
346 bromide and visualised under UV light. A 100-bp DNA ladder was run in parallel with the
347 samples.

348 The parasite load was determined by means of quantitative PCR. For the amplification reaction,
349 2µl of a 1/100 diluted DNA sample was added to 8µl mix containing 5µl KAPA Probe Fast qPCR
350 master mix (2x) (Kapa Biosystems, Wilmington, MA), 0.4 µM of each primer and 0.25µM of the
351 appropriate probe. The tubes were placed in the 72 sample rotor of the instrument (Rotor Gene
352 3000, Qiagen) and the reaction with the following conditions was initiated: 95°C for 3 minutes
353 followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Each run contained a
354 standard curve, a no-template-control and a negative control.

355

356

357 **Results**358 **Structures of the compounds**

359 Benzoxaborole compounds from 4 different chemical classes that had shown anti-parasitic
360 activity in the *P. falciparum*, *T. brucei*, *T. cruzi* or *L. donovani* screens were selected from the
361 library for screening against CL causing species. Some of the subclasses tested are shown in
362 Table 1 and include benzoxaborole 6-carboxamides (D), benzoxaborole-5-carboxamides (B),
363 pyrazole 6-carboxamides (C) and benzoxaborininols (E) in which the 5-ring containing the boron
364 atom is replaced by a 6-ring structure.

365 ***In vitro* anti-leishmanial activity**

366 Twenty-five compounds were screened against intracellular amastigotes. LSH001, LSH003,
367 LSH023, LSH024 and LSH025 were the only five compounds that showed activity against at least
368 one Old World (*L. major*, *L. tropica* and *L. aethiopica*) and one New World (*L. mexicana* and *L.*
369 *panamensis*) species with an EC₅₀ value below 30 µM (Table 5). These five test compounds were
370 most active against *L. tropica* with an EC₅₀ value below 5 µM followed by *L. major* with EC₅₀
371 values in the same range. *L. mexicana* was the least susceptible species with EC₅₀ values ranging
372 from 9 to 22 µM. For most tested compounds, the EC₅₀ value against *L. mexicana* was higher
373 than 30 µM, the highest concentration tested suggesting low activity of the compound.

374 Amphotericin B, included as positive control, had a high activity with EC₅₀ values ranging from
375 0.049 to 0.685 µM, indicating a tenfold difference in sensitivity between *L. major/L. tropica* and
376 *L. mexicana*. Miltefosine, the other control drug, was less active than amphotericin B with EC₅₀
377 values ranging from 7 to 45 µM and 10 to 35 µM respectively.

378 At this stage, it was decided to advance all compounds with potent ($EC_{50} < 5\mu M$) and/or
379 moderate activity ($5\mu M < EC_{50} < 25\mu M$) against at least one Old World and one New World
380 *Leishmania* species. Eight compounds (LSH026-034) with promising activity against other
381 *Leishmania* species (DNDi, unpublished data) were also included in further assays.

382 **Physicochemical properties**

383 An initial computational screening of the test compounds was conducted to evaluate
384 permeation related physicochemical properties i.e. the molecular weight, the presence of H-
385 bond donors or acceptors and the aqueous solubility. The partition coefficient was determined
386 experimentally. It was found that the benzoxaborole test compounds had appropriate
387 physicochemical profiles for skin permeation (Table 6), i.e. a molecular weight below 500
388 g/mol, a log D (at pH 7.4) between 1-3 (except for LSH002 (logD = 0.44) and LSH032 (logD =
389 0.88)) and no more than 2 H-bond donor groups.

390 **Intrinsic permeability**

391 The MDCK-MDR1 assay was performed to identify P-glycoprotein (Pgp) substrate and to
392 evaluate passive permeability of the test compounds across simple epithelia such as that of the
393 intestine (35). The test compounds generally demonstrated high passive permeability (Table 7)
394 in the assay with values ranging from 247-688 nm/s (32) except for compound LSH002 that
395 showed a low intrinsic permeability of 32.5 nm/s. Further, only one compound (LSH002; AQ
396 value: 0.492) exceeded the cut-off value (>0.3) for absorptive quotient indicating it was a
397 potential substrate for the efflux transporter Pgp. For comparison, Amprenavir, the positive
398 control included as a known Pgp substrate afforded an AQ value of 0.846. Interestingly, the

399 most active compounds during in vitro susceptibility studies all showed permeability values
400 above 300 nm/s and were no Pgp substrates.

401 Previous research suggested an enhanced permeability of hydrophilic compounds in
402 *Leishmania*-infected skin (20). Compounds LSH002 and LSH032 were therefore included in
403 further assays despite their less favourable physicochemical properties and/or intrinsic
404 permeability.

405 **Dermal stability, binding and permeability**

406 *Stability in skin supernatant.* An initial rapid drug degradation of the test compounds was
407 observed in skin supernatant (Figure 2) during the first 30 minutes, followed by slower drug
408 metabolism. After two hours, compound recovery was 25 to 60% with LSH001, 002, 024, 025,
409 028, 031, 032, 034 being moderately stable (% remaining: 25-44) and LSH003, 023, 026, 027,
410 029, 030, 033, 034 being most stable with 45-75% test compound remaining. The two paraben
411 compounds, ethyl and propyl paraben, known substrates for skin esterases, were observed to
412 break down very quickly in presence of the skin supernatant. The recovery of these labile
413 compounds was 10.9 and 0% respectively after 2 hours of incubation.

414 *Drug binding to skin components.* A binding assay showed large variations in unbound fractions
415 among the benzoxaboroles; unbound fractions from 34% to 92% were observed (Table 8). Only
416 2 compounds had a high free fraction of 85% or more comprising LSH001 and LSH026. The
417 majority of the compounds has a free fraction between 50 and 85% and finally LSH003 and
418 LSH023 with the lowest free fractions of 44 and 34% respectively.

419 *RHE permeability.* RHE was used to evaluate the passive permeability of the test compounds
420 across multiple layered membranes more representative of skin. The permeation of LSH002
421 was statistically significant higher compared to LSH001, LSH029 and LSH033 (one-way ANOVA,
422 $p < 0.05$). As anticipated, the high permeability hydrophilic control caffeine showed the highest
423 permeation, which was significantly higher when compared to all the test compounds and
424 testosterone (lower permeability, hydrophobic control) after 6 hours (one-way ANOVA; $p <$
425 0.05). When ranking the cumulative amount permeated over 6 hours, the rank order from high
426 to low was as follows: caffeine > LSH002 > LSH003 > LSH023 > testosterone > LSH024 > LSH033
427 > LSH001 > LSH029.

428 Both caffeine and LSH002 are more hydrophilic compounds as indicated by their low log D of -
429 0.08 and 0.44, respectively. The vehicle in which all drugs were applied was ethanol-Miglyol 840
430 (1:9). LSH002 even though in solution, might have been closer to saturation exhibiting a higher
431 thermodynamic activity compared to the other test compounds with a higher log D. The higher
432 permeation exhibited by LSH002 could also involve the higher affinity of this compound for the
433 RHE compared to the lipophilic vehicle thereby stimulating its preferential partitioning into the
434 membrane.

435 Based on the overall data set collected, it was decided to select three compounds (LSH001,
436 LSH002 and LSH003) for further study. LSH001 was included because it showed potent anti-
437 leishmanial activity and was representative of a lipophilic compound, despite lower
438 permeability, that may prove helpful for formulation and skin disposition. LSH002 was included
439 due to its higher solubility in water and hence a control for disposition in the skin permeation

440 assay and LSH003 was selected because it was active against the 5 *Leishmania* species tested
441 and it was ranked second with regards flux in the permeation assay.

442 **Dermal disposition**

443 The objective of the first permeation study in mouse skin was to verify the rank order of the
444 three selected compounds and compare them with the results obtained from the previous
445 permeation experiment where a RHE model was used (Table 9). Therefore, the experimental
446 conditions and drug formulations were similar to the RHE experiment. The results are shown in
447 Figure 3 and indicate that the rank order LSH002 > LSH003 > LSH001 is maintained when using
448 BALB/c mouse skin instead of the RHE membrane. Furthermore, the permeation of LSH002
449 through BALB/c mouse skin was significantly higher compared to LSH001, LSH003 and
450 testosterone (one-way ANOVA, $p < 0.05$).

451 A second permeation study using BALB/c mouse skin aimed to assess the permeation and skin
452 disposition of the compounds after application of a low volume of a 1% solution of test
453 compound in ethanol-propylene glycol (E-PG) (1:1) solution ($28 \mu\text{L}/\text{cm}^2$) comparable with the
454 formulation intended for use in the murine CL model. Permeation (Table 8) was statistically
455 higher for LSH001 and LSH003 ($p < 0.05$, one-way ANOVA) compared to LSH002. The rank order
456 for flux was LSH001 > LSH003 > LSH002. Of note, in the E-PG formulation the more hydrophobic
457 compounds (LSH001 and LSH003) achieved greater permeation than LSH002. There was no
458 difference observed in the lag time for the different compounds (ANOVA; $p > 0.05$).

459 A skin disposition study (Figure 4) was conducted to compare the amount of test compound
460 that either: remained on the surface of the skin, retained within the dermal layers or had

461 permeated through the skin. Whilst there was no statistical significant difference between the
462 amounts of compounds that had permeated over 24 hours, the amount of LSH001 in the skin
463 was significantly higher in comparison to LSH002 and LSH003 (one-way ANOVA; $p < 0.05$). The
464 mass balance for the total compound recovering was 84%, 87% and 114% for LSH001, LSH002
465 and LSH003, respectively indicating excellent mass balance was achieved for all compounds
466 across the compartments.

467 ***In vivo* anti-leishmanial activity**

468 After 10 days of topical application of the three selected compounds to the closed nodules,
469 LSH001 halted the lesion size progression and the lesions in this group were statistically smaller
470 compared to the vehicle control group (One-way ANOVA, $p < 0.05$) whereas no lesion size
471 reduction was observed for LSH002 or LSH003 (Figure 5 (A)). The lesion sizes and parasite
472 burden per lesion of the groups 3 days after the last drug administration are shown in Figure 5
473 (B). The parasite load in the group receiving topical LSH001 is slightly lower than in the other
474 topically treated groups however there is no statistically significant difference (One-way
475 ANOVA, $p > 0.05$).

476 Whilst the primary aim of this work was to investigate the potential of benzoxaboroles for
477 topical treatment for CL, the *in vitro* ADME data suggest good overall permeability. Previous
478 studies of the benzoxaboroles as orally active drugs for HAT suggested good oral bioavailability
479 for this class. Therefore, we administered the three test compounds LH001, LH002 and LH003
480 orally to CL infected mice. A significant reduction of lesion size was seen for the groups
481 receiving LSH003 by the oral route compared to the relevant control group (One-way ANOVA,

482 $p < 0.05$). This was also reflected in parasite load as the number parasites per lesion was
483 statistically significant lower compared to the untreated control group (One-way ANOVA,
484 $p < 0.05$).

485 For AmBisome[®], the positive control, a statistically significant reduction in lesion diameter and
486 parasite load was observed compared to the control group ($p < 0.05$) except for the LSH001
487 topical and LSH003 oral groups ($p > 0.05$). This was expected as per previous reports describing a
488 reduction of both lesion size and parasite burden (36).

489

490 **Discussion**

491 Topical treatment for a dermatological infection limited to the more superficial layers of the
492 skin, offers an attractive alternative to the currently used routes of administration for CL
493 treatment as it (i) allows local drug targeting directly to the infection site, (ii) offers the
494 potential to limit adverse effects, (iii) is not invasive and (iv) is easy to apply by the patient. A
495 systematic approach to the identification of potential lead compounds to progress to clinical
496 trials is still lacking. The goal of this work was to explore a novel approach to identify promising
497 compounds for the treatment of CL.

498 The benzoxaborole class of anti-parasitics has demonstrated efficacy across multiple parasitic
499 disease targets including the *Leishmania spp.* DNDI-6148 is at the preclinical stage of
500 development for treatment for visceral leishmaniasis (37) and oxaborole SCYX-7158 is now in
501 phase 2 clinical trials for the treatment of HAT (37). The goal of these programs was to identify
502 orally active treatments of these systemic parasitic infections.

503 For successful therapeutic activity *in vivo* in CL, a drug requires both potent anti-leishmanial
504 activity and an ability to permeate biological membranes in order to reach the *Leishmania*
505 parasites in the dermal layer of the skin, a process that is impacted by both the physicochemical
506 properties of the drug and the route of administration.

507 Several criteria limit delivery of drugs through the skin; drugs with a molecular weight of < 500
508 g/mol (38), a partition coefficient between 1 and 3 (39, 40) , a low melting point (< 200°C) (41),
509 aqueous solubility >1 mg/ml (42) and less than 2 H-bond donor groups (43) are more likely to
510 permeate. Topically applied drugs also undergo relatively little enzymatic degradation
511 compared to orally administered drugs that need to pass a monolayer of intestinal epithelium
512 and have low hepatic first-pass metabolism before it reaches the blood circulation to allow it to
513 distribute to the skin (40).

514 Whilst each layer of the skin is a potential hurdle to drug permeation, it is the outer layer of the
515 skin, the stratum corneum, that is a highly restrictive permeability barrier formed of 10-15
516 layers of dead keratinized cells imbedded in an intercellular lipid mixture organised in bilayers
517 (44, 45). This inherent difference between bio-membranes governs the preferential
518 permeability of certain drugs (40).

519 Previously reported strategies for developing topical treatments for CL have focussed solely on
520 formulation optimisation as means to increase treatment efficacy, whereas we wish to identify
521 compounds with intrinsic properties consistent with topical administration. To achieve this
522 objective we systematically evaluated physiologically-based pharmacokinetic parameters and
523 aimed to correlate these to the physicochemical properties of the compounds. A diverse set of

524 benzoxaboroles associated with good drug-like properties in previous anti-parasitic programs
525 was selected. Compounds were assessed for their likely intrinsic activity against old and new
526 world CL species by measuring the *in vitro* activity against the intracellular amastigote form
527 using a previously reported assay (46).

528 Dermal drug-like properties were characterised by comparing physicochemical properties, *in*
529 *vitro* permeability through MDCK, and RHE models and stability in skin homogenate.
530 Subsequently promising compounds were advanced to whole skin permeability, binding and
531 disposition evaluation. This strategy of selection was employed to advance the most promising
532 compounds to the more complex assays. Ultimately, this strategy identified 3 compounds each
533 with unique features for evaluation in a murine model of CL.

534 Initially, five benzoxaboroles 6-carboxamides showed broad range activity against CL causing
535 species. To complement this intrinsic activity, *in vitro* membrane permeability assays were
536 employed to assess each compound's ability to cross a cellular barrier. Previously, the MDCK-
537 MDR1 Transwell assay was successfully utilised to classify compounds with a potential high
538 permeability across the gut when $P_{app} A \rightarrow B_{+GF918} > 50$ nm/s (31) or the blood brain barrier when
539 the $P_{app} A \rightarrow B_{+GF918} > 150$ nm/s and the compound is a non-PgP substrate (47). Whilst for dermal
540 permeation no clear selection criteria were found in literature, our test compounds generally
541 exhibited high permeability with a $P_{app} A \rightarrow B_{+GF918} > 200$ nm/s, except for LSH002 ($P_{app} A \rightarrow B_{+GF918}$
542 = 32.5 nm/s). Furthermore, the MDCK-MDR1 assay allowed us to identify potential substrates
543 of the P-glycoprotein (PgP) efflux transporter (48-50) which is helpful considering that these
544 compounds may suffer reduced fraction absorbed following oral delivery (51, 52) but may also

545 demonstrate reduced ability to penetrate macrophages. This is important because *Leishmania*
546 parasites survive and divide inside macrophages meaning that Pgp substrate drugs might
547 potentially be less active compared to drugs that are not Pgp substrates as efflux would
548 attenuate entry into macrophages (53, 54). In fact, reports of inactivity of antimonial drugs
549 against *L. donovani* in patients were linked to upregulation of Pgp transporters in the host cells,
550 leading to low concentrations of drug in the macrophages and thus disease progression (55). In
551 our set of test compounds, only LSH002 showed an absorption quotient higher than 0.3
552 indicating it potentially is a Pgp substrate (31).

553 Moving on from the cellular models of permeation, the permeability of the test compounds
554 was further evaluated in complex RHE that has shown ability to predict dermal permeation (56)
555 allowing us to further rank order our test compounds. The hydrophilic compounds, caffeine and
556 LSH002, showed highest permeation in this model. Caffeine and LSH002 were the most
557 hydrophilic compounds amongst the test compounds as was indicated by their log D value of -
558 0.08 and 0.44 respectively. Hence, LSH002 even though in solution, might have been closer to
559 saturation in the ethanol-Miglyol 840 (1:9) vehicle exhibiting a higher thermodynamic activity
560 compared to the test compounds with a higher log D. The second highest permeation was
561 observed for LSH003 the test compound that also showed good *in vitro* anti-leishmanial activity
562 against all five *spp*. LSH001, also active *in vitro* against all *Leishmania spp*, showed a slightly
563 lower permeation than testosterone the lipophilic control drug.

564 When evaluating the permeation of these three compounds in BALB/c mouse skin using the
565 same experimental design, the overall permeation and thus flux of the test compounds and

566 testosterone were lower compared to the permeation through RHE (one-way ANOVA, $p < 0.05$)
567 (Table 6). Several studies have indicated that RHE is more permeable than animal and human
568 skin (57-59). The rank order of the test compound's permeation through mouse skin was the
569 same as for the RHE (LSH002 > LSH003 > LSH001) and more importantly, the permeation of all
570 test compounds was higher than that of testosterone.

571 We next explored the metabolic stability of the benzoxaboroles in both liver-based and skin-
572 based assays. Degradation of drugs in the skin has been reported (60-62) with the main site of
573 activity situated in the epidermis (63). We used the supernatant of skin homogenate to
574 determine the drug stability and observed that all benzoxaborole test compounds showed a
575 higher stability compared to the paraben compounds that are known substrates for skin
576 esterases and are therefore expected to breakdown (64). The fraction of parent compound
577 remaining after 2h of incubation was relatively similar for all compounds ranging from
578 approximately 30 to 60%. The skin homogenate was prepared using full-thickness BALB/c
579 mouse skin as opposed to epidermal membranes alone. Epidermal membranes exhibited
580 reduced enzymatic activity compared to full-thickness skin (65), possibly due to the exposure to
581 heat required to separate epidermal and dermal membranes. Furthermore, the *in vivo* efficacy
582 study will be conducted in female BALB/c mice and thus full-thickness mouse skin was used to
583 assure consistency between the *in vitro* – *in vivo* data set. During the preparation of our
584 homogenate intracellular enzymes might have been released contributing to the breakdown of
585 drugs, in which case these results represent an overestimation of drug metabolism (62). How
586 these results compare to human skin is unclear but a study comparing paraben breakdown in
587 rat and human skin, observed a higher metabolism, in the order of magnitudes, for rat skin

588 indicating that the breakdown in human skin is expected to be lower as compared to the results
589 obtained here (66).

590 Drug binding to skin proteins can also result in the inability of the drug to reach or distribute to
591 its target; it is the unbound (free) fraction of the drug in the dermis that is pharmacologically
592 active as it can passively permeate into the macrophage and from there into the parasite (67).
593 After incubation in skin homogenate, our test compounds exhibited a range of unbound
594 fractions. A certain level of drug-skin binding is desirable to establish a depot effect leading to
595 slow release of the drug from the skin into the macrophage and *Leishmania* parasite. As the
596 unbound fractions across a membrane are in equilibrium, drug being taken up by the
597 macrophage will cause drug bound to skin components to be released and become available for
598 uptake into the macrophage. Moreover, the skin binding could prevent systemic exposure and
599 therefore preliminary drug metabolism and excretion.

600 Prior to *in vivo* evaluation, the skin disposition of the compounds was evaluated using BALB/c
601 mouse skin under real-life conditions e.g. limited volume of a 1% (w/v) test compound
602 formulation. This showed a lower permeation for LSH002 compared to LSH001 and LSH003 in
603 contrast to its higher permeation shown in the RHE. This is likely due to the change in vehicle
604 and thus saturation therein. LSH001 and LSH003 were applied as suspensions with a maximal
605 thermodynamic activity, while LSH002 was applied as a solution at about 80% of saturation and
606 thus a suboptimal thermodynamic driving force. Also due to its hydrophilic nature, LSH002 is
607 likely to have a higher affinity for the ethanol-PG vehicle compared to the skin causing the drug
608 to remain in the vehicle on the skin surface. In addition, our mass balance data showed a

609 significantly lower drug fraction in the skin for both LSH002 and LSH003 compared to LSH001
610 (one-way ANOVA; $p < 0.05$). For LSH001 about half of the applied drugs had permeated into the
611 skin. LSH001 has a high log D which is expected to facilitate partitioning and diffusion into the
612 stratum corneum. A high log D, however, is unfavourable for the permeation into the dermis.

613 When evaluating the activity of these test compounds *in vivo*, LSH001 applied topically was able
614 to halt the lesion growth, which suggests that the drug was able to permeate through the SC
615 and reach the parasites situated in the lower epidermis and dermis. LSH003 administered
616 orally, significantly reduced the lesion size and parasite burden compared to the LSH001 and
617 LSH002 oral groups. This non-healing BALB/c model is a rigorous test for drugs because (i) upon
618 infection with *Leishmania* parasites, the mice develop fulminating infections with ulcers that
619 quickly progress to death if left untreated (68), and (ii) the drugs were only applied after
620 establishment of the lesions. For this model lesion size reduction or suppression of lesion
621 growth is regarded as a promising result (68).

622 The determination of efficacy of the topical formulations can be difficult to gauge as the mice
623 are able to remove the formulation by licking the site. For LSH001, there was no change in
624 lesion progression when the compound was administered orally. This suggests that the drug
625 that permeated the skin exerts the suppression of nodule growth observed upon topical
626 application of the same agent. This correlates with the data obtained from the *in vitro*
627 permeation experiment using BALB/c mouse skin. Not only did LSH001 show a higher
628 permeation compared to LSH002 and LSH003, the mass balance study also showed a
629 statistically higher concentration of LSH001 in the skin compared to the two other test

630 compounds. Of the three *in vivo* tested compounds, LSH001 also exhibited the highest unbound
631 fraction. It could be hypothesised that even for topical compounds it is beneficial to have a high
632 unbound fraction in order to exert anti-leishmanial activity as opposed to binding to skin.

633 LSH001 suppressed nodule growth when applied topically whereas oral administration with the
634 same agent did not affect lesion size and vice versa for LSH003 whereby oral administration
635 reduced the lesion size but topical administration had no effect. Since LSH001 and LSH003,
636 exhibited the same *in vitro* activity against *L. major*, it is thus suggested that the difference in
637 efficacy upon oral administration is due to pharmacokinetic variations between LSH001 and
638 LSH003.

639 **Conclusions**

640 Previously, the process of drug development for CL mainly focussed on drug activity testing and
641 formulation optimisation. Current *in vitro* models to test anti-leishmanial activity rely on 2D
642 culture systems that demonstrate activity against the intracellular parasite but correlate poorly
643 with results obtained in animal models (69). This “disconnect” is likely to be caused by the
644 oversimplification of the *in vitro* model that is unable to account for pharmacokinetic drug
645 barriers that occur *in vivo*.

646 We have shown that a more complete evaluation of a drug candidate is established by
647 incorporating physiologically-based pharmacokinetic assays in our drug discovery, leading to an
648 improved selection of lead candidates, which is essential to improve the likelihood of a success
649 of clinical candidates (70, 71).

650 Furthermore, this step-wise approach allows evaluation of the test compounds at each stage
651 enables input from medicinal chemistry to alter the core molecule to optimise physicochemical
652 properties to increase distribution and specificity of the drug in the skin at an early stage of
653 development.

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664 **Transparency declarations**

665 None to declare

666

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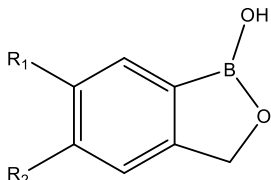
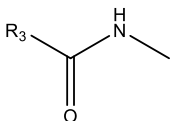
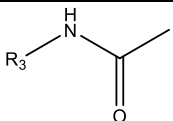
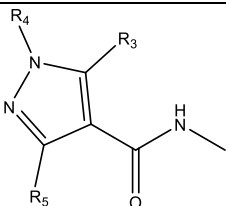
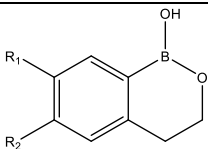
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- 877

878 **Tables**

879 Table 1. The general structure of benzoxaboroles (A) and subclasses: benzoxaborole 6-
880 carboxamides (D), benzoxaboroles 5-carboxamides (B), pyrazole 6-carboxamides (C),
881 benzoxaborininols (E).

General benzoxaboroles structure		
		
Chemical sub class	Modification	Compound ID
Benzoxaborole 6-carboxamide	R1 	LSH006, LSH009, LSH010, LSH011, LSH012, LSH015, LSH016, LSH019, LSH020, LSH021, LSH023, LSH024, LSH025
Benzoxaborole 5-carboxamide	R2 	LSH002, LSH031
Pyrazole 6-carboxamides	R1 	LSH022, LSH027, LSH028, LSH029
benzoxaborininole		LSH001, LSH033
Other		LSH004, LSH005, LSH007, LSH013, LSH017, LSH018, LSH034

882

883 Table 2. Summary of the experimental conditions for the different permeation experiments.

Permeation experiment	Compounds	Donor vehicle	Concentration (µg/ml)	Volume/ skin surface (µl/cm ²)
RHE 1	LSH001; LSH003; LSH011; LSH012; LSH023; LSH024; LSH029; LSH034; caffeine; testosterone	Ethanol – Miglyol 840 (1:9) Except for caffeine	100	300
FDC 1	Mix1: LSH001; LSH002 Mix2: LSH003; LSH034	Ethanol – Miglyol 840 (1:9)	100	300
FDC 2	LSH001; LSH002; LSH003	Ethanol – PG (1:1)	10 000 (1% w/v)	28.4

884

885 Table 3. Summary of the different *in vivo* experimental groups with their treatment regimen.

Group	Formulation	Active compound	Vehicle	Administration route	Treatment regimen
1	Untreated control	None	None	None	None
2	AmBisome [®]	Amphotericin B	Dextrose 5%	IV	25mg/kg/b.i.d, 5 doses
3	Leshcutan [®]	Paromomycin sulphate 15%	Methylbenzethonium chloride 12% in vaseline	Topical	0.1ml 2/day for 10 days
4	Vehicle control	N/A	PG/Ethanol (1:1)	Topical	2x50µl/day for 10 days
5	Topical formulation 1	LSH001	Saturated drug solution in PG/Ethanol (1:1)	Topical	2x50µl/day for 10 days
6	Topical formulation 2	LSH002			
7	Topical formulation 3	LSH003			
8	Oral formulation 1	LSH001	Standard suspended vehicle	Oral	2x25mg/kg/day for 10 days
9	Oral formulation 2	LSH002			
10	Oral formulation 3	LSH003			

886

887

888 Table 4. The sequences of the primer and probes used in the PCR and qPCR reactions.

	Gene	Primer/probe	Primer Sequence
<i>Leishmania</i> species	18S rDNA (170-bp)	Forward primer	5'-C CAA AGT GTG GAG ATC GAA G-3'
		Reverse primer	5'-GGC CGG TAA AGG CCG AAT AG-3'
		Probe	5'-6FAM ACCATTGTAGTCCCACTGC-NFQ-MGB

889

890 Table 5. Activity of benzoxaborole compounds against intracellular *Leishmania* amastigotes
891 (EC₅₀ values (μM) and 95% CI, n=number of experiment repeats).

Compound	n	<i>L. tropica</i>	<i>L. major</i>	<i>L. aethiopia</i>	<i>L. mexicana</i>	<i>L. panamensis</i>
Amphotericin B	1	0.066 (0.062-0.070)	0.043 (0.037-0.049)	0.115 (0.107-0.122)	0.430 (0.394-0.460)	0.143 (0.131-0.156)
	2	0.083 (0.078-0.089)	0.049 (0.043-0.056)	0.107 (0.096-0.119)	0.685 (0.553-0.692)	0.115 (0.093-0.142)
Miltefosine	1	19.99 (17.40-22.97)	44.85 (22.02-77.28)	7.79 (6.20-9.78)	31.04 (28.56-33.73)	19.98 (16.17-24.69)
	2	9.44 (7.78-11.45)	26.58 (21.30-33.15)	7.95 (7.26-8.69)	45.86 (36.61-57.45)	23.11 (20.41-26.18)
LSH001	1	2.01 (1.52-2.67)	4.26 (2.97-6.11)	22.10 (15.07-32.41)	23.04 (15.99-33.19)	18.82 (14.08-25.14)
	2	3.12 (2.38-4.09)	7.61 (5.48-10.57)	26.83 (19.40-37.11)	16.94 (9.62-29.83)	13.96 (10.06-19.44)
LSH002	1	14.96 (11.38-19.67)	16.52 (11.56-23.61)	> 30	> 30	> 30
LSH003	1	2.46 (1.78-3.41)	3.93 (3.32-4.64)	11.12 (7.67-16.13)	18.94 (10.78-33.29)	8.09 (6.56-9.96)
	2	3.94 (2.96-5.25)	3.10 (2.25-4.26)	> 30	> 30	19.05 (15.03-24.16)
LSH004	1	16.08 (13.70-18.80)	-	29.97 (19.04-47.16)	> 30	> 30
LSH005	1	6.81 (5.84-7.94)	-	21.25 (13.18-34.26)	> 30	> 30
LSH006	1	> 30	-	25.36 (15.88-40.50)	> 30	> 30
LSH007	1	5.71 (4.39-7.43)	-	27.18 (17.16-43.04)	> 30	> 30
LSH008	1	> 30	-	> 30	> 30	> 30
LSH009	1	3.08 (2.51-3.79)	-	17.66 (12.10-25.76)	> 30	> 30
LSH010	1	6.23 (5.49-7.06)	> 30	11.71 (7.22-19.00)	> 30	> 30
LSH011	1	2.31 (1.73-3.08)	9.92 (8.49-11.59)	> 30	> 30	> 30
LSH012	1	24.61	9.52	> 30	> 30	> 30

		(14.31-42.30)	(6.80-13.32)			
LSH013	1	> 30*	> 30	> 30	> 30	> 30
LSH014	1	5.91* (4.63-7.54)	4.15 (3.42-5.04)	> 30	> 30	29.59 (20.59-42.53)
LSH015	1	6.92 (4.95-9.66)	> 30	> 30	> 30	> 30
LSH016	1	5.40 (4.02-7.26)	> 30	21.84 (14.60-32.66)	> 30	> 30
LSH017	1	> 30	> 30	> 30	> 30	> 30
LSH018	1	> 30	> 30	> 30	> 30	> 30
LSH019	1	21.01 (4.07-108.4)	> 30	> 30	> 30	> 30
LSH020	1	> 30	> 30	> 30	> 30	> 30
LSH021	1	28.81 (17.03-48.74)	> 30	> 30	> 30	> 30
LSH022	1	> 30	> 30	> 30	> 30	> 30
LSH023	1	1.19* (0.78-1.80)	1.57 (1.17-2.10)	23.05 (10.09-52.62)	6.31 (4.18-9.54)	2.98 (2.28-3.90)
LSH024	1	4.72* (3.31-6.74)	13.96 (11.52-16.91)	> 30	> 30	22.34 (17.66-28.25)
LSH025	1	2.21* (1.51-3.25)	5.93 (5.08-6.92)	> 30	25.39 (15.81-40.78)	15.85 (12.85-19.56)

892

893

Table 6. Physicochemical properties of benzoxaborole compounds.

Compound	Molecular weight* (g/mol)	H bond donor/acceptor*	Aqueous solubility* (µg/ml)	Log D (pH 7.4)**
Ideal skin permeant	< 500	< 3		1-3
LSH001	387	2/5	9	> 2.63
LSH002	421	2/5	37	0.44±0.06
LSH003	321	2/7	165	2.18±0.08
LSH023	334	2/5	103	2.45±0.04
LSH024	368	2/6	45	2.16±0.07
LSH026	306	2/4	22	1.86±0.07
LSH027	325	2/8	103	1.53***
LSH028	334	2/6	53	1.86±0.02
LSH029	393	2/11	14	1.95±0.10
LSH030	373	2/5	13	1.94±0.06
LSH032	386	2/5	11	0.88±0.15
LSH033	400	2/5	7	1.70±0.15

894 * Data obtained using ChemBio 3D Ultra 13.0 modeling software

895 ** Experimental data, Mean±SD, n=3

896 *** Experimental data, n=1

897

898

899 Table 7. The P_{app} values with and without GF918 and the absorptive quotient (AQ) for the
900 MDCK-MDR1 assay.

Compound	P_{app} (nm/s)	$P_{app} + GF918$ (nm/s)	AQ
Amprenavir	58.3	378	0.846
Propranolol	395	441	0.104
LSH001	583	599	0.027
LSH002	16.5	32.5	0.492
LSH003	626	635	0.014
LSH023	605	593	-0.020
LSH024	236	314	0.248
LSH025	322	349	0.077
LSH026	652	655	0.005
LSH027	209	247	0.154
LSH028	397	424	0.064
LSH029	229	268	0.146
LSH030	436	524	0.168
LSH032	232	327	0.291
LSH033	404	482	0.162
LSH034	543	538	-0.009

901

902 Table 8. Fractions of unbound compound and remaining compound after 2 hours incubation
903 with mouse skin supernatant (protein content 2.5 mg/ml).

Compound	% unbound	% remaining test compound
ethyl paraben		10.9
propyl paraben		0.0
LSH001	87	44.1
LSH002	59	28.0
LSH003	44	51.0
LSH023	34	50.2
LSH024	50	35.0
LSH025	66	34.3
LSH026	92	50.8
LSH027	62	53.8
LSH028	60	41.5
LSH029	79	64.2
LSH030	67	46.2
LSH032	57	41.7
LSH033	65	46.3

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908 Table 9. The permeation parameters: flux and lag time when using RHE and BALB/c mouse skin
 909 under same conditions and BALB/c mouse skin when applying a low volume (mean±sd; n=3
 910 except for * where n=2).

	RHE	BALB/c	BALB/c Low volume
Testosterone			
flux (ng/cm ² /h)	28.0±0.8	2.2±0.8	
lag time (h)	0.7±0.1	1.1±0.6	
LSH001			
flux (ng/cm ² /h)	21.8±0.1	6.6±0.3*	88.7±8.8
lag time (h)	2.2±0.1	2.4±0.4	2.7±0.5*
LSH002			
flux (ng/cm ² /h)	143.9±44.2	35.8±0.9*	13.5±8.7*
lag time (h)	1.7±0.8	2.8±0.3	2.7±0.7
LSH003			
flux (ng/cm ² /h)	45.1±5.9	8.0±1.5*	71.8±18.0
lag time (h)	2.1±0.1	2.5±0.3	2.7±0.9

911

912 **Figure Legends**

913 **Figure 1. Drug delivery for CL. (A) Progression pathway during lead optimization of drugs as**
914 **potential topical treatment for CL. (B) Histology of BALB/c mouse skin infected with *L. major*.**

915 (A) Schematic of the route of the active drug through *Leishmania* infected BALB/c mouse skin
916 before reaching (B) the *Leishmania* amastigotes situated in phagolysosome of dermal
917 macrophages.

918 **Figure 2. *In vitro* stability of test compounds in skin homogenate.** The remaining fraction (%)
919 of test compound left in supernatant with a protein content of 2.5mg/ml as a function of time
920 (mean±SD, n=3)

921 **Figure 3. *In vitro* permeation through BALB/c mouse skin.** The cumulative amount permeated
922 in time for LSH001, LSH002 and LSH003 using Franz diffusion cells (mean±SD, n=3).

923 **Figure 4. Skin disposition evaluation.** The amounts of benzoxaboroles that permeated or were
924 found in and on the skin expressed as percentage of the dosage retrieved.

925 **Figure 5. *In vivo* anti-leishmanial activity of benzoxaboroles upon oral and topical application.**

926 (A) The progression of the main lesion size (measured using digital callipers) per group in
927 function of time post infection (n=6, mean±SD); (B) The average number of amastigotes found
928 per lesion as analysed by qPCR and the average lesion size per group 3 days after the end of the
929 treatment (mean±SD, n=5).

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