

# Accepted Manuscript

Novel benzoxaborole, nitroimidazole and aminopyrazoles with activity against experimental cutaneous leishmaniasis

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PII: S2211-3207(18)30185-4

DOI: <https://doi.org/10.1016/j.ijpddr.2019.02.002>

Reference: IJPDDR 291

To appear in: *International Journal for Parasitology: Drugs and Drug Resistance*

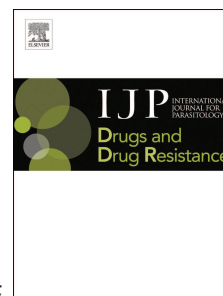
Received Date: 14 December 2018

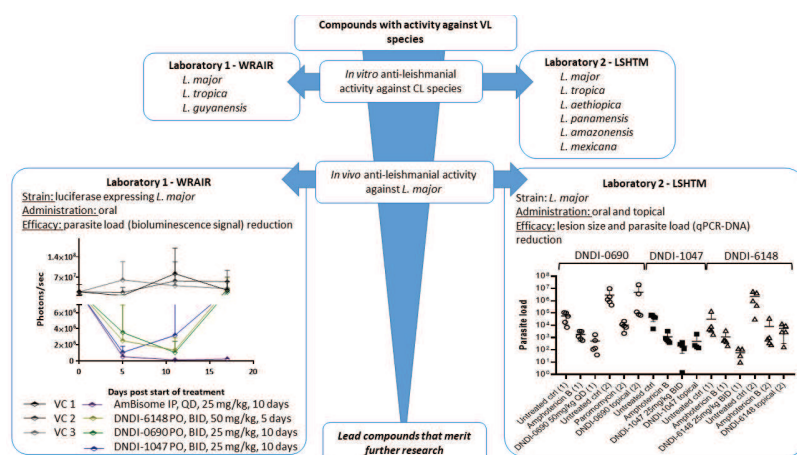
Revised Date: 6 February 2019

Accepted Date: 12 February 2019

Please cite this article as: Van Bocxlaer, K., Caridha, D., Black, C., Vesely, B., Leed, S., Sciotti, R.J., Wijnant, G.-J., Yardley, V., Braillard, Sté., Mowbray, C.E., Ioset, J.-R., Croft, S.L., Novel benzoxaborole, nitroimidazole and aminopyrazoles with activity against experimental cutaneous leishmaniasis, *International Journal for Parasitology: Drugs and Drug Resistance* (2019), doi: <https://doi.org/10.1016/j.ijpddr.2019.02.002>.

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4 **Journal suggestion:** International Journal for Parasitology: Drugs and Drug Resistance

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22

23 **Abstract:**

24 Objectives: Drugs for Neglected Diseases *initiative* (DNDi) has identified three chemical lead series,  
25 the nitroimidazoles, benzoxaboroles and aminopyrazoles, as innovative treatments for visceral  
26 leishmaniasis. The leads discovered using phenotypic screening, were optimised following disease-  
27 and compound-specific criteria. Several leads of each series were progressed and preclinical drug  
28 candidates have been nominated. Here we evaluate the efficacy of the lead compounds of each of  
29 these three chemical classes in *in vitro* and *in vivo* models of cutaneous leishmaniasis.

30 Methods: The *in vitro* activity of fifty-five compounds was evaluated against the intracellular  
31 amastigotes of *L. major*, *L. aethiopica*, *L. amazonensis*, *L. panamensis*, *L. mexicana* and *L. tropica*. The  
32 drugs demonstrating potent activity ( $EC_{50} < 5\mu\text{M}$ ) against at least 4 of 6 species were subsequently  
33 evaluated *in vivo* in different *L. major* – BALB/c mouse models using a 5 or 10-day treatment with  
34 either the oral or topical formulations. Efficacy was expressed as lesion size (measured daily using  
35 callipers), parasite load (by quantitative PCR – DNA) and bioluminescence signal reduction relative to  
36 the untreated controls.

37 Results: The selected drug compounds (3 nitroimidazoles, 1 benzoxaborole and 3 aminopyrazoles)  
38 showed consistent and potent activity across a range of *Leishmania* species that are known to cause  
39 CL with  $EC_{50}$  values ranging from 0.29 to  $18.3\mu\text{M}$ . In all cases, this potent *in vitro* antileishmanial  
40 activity translated into high levels of efficacy with a linear dose-response against murine CL. When  
41 administered at 50mg/kg/day, DNDI-0690 (nitroimidazole), DNDI-1047 (aminopyrazole) and DNDI-  
42 6148 (benzoxaborole) all resulted in a significant lesion size reduction (no visible nodule) and an  
43 approximate 2-log-fold reduction of the parasite load as measured by qPCR compared to the  
44 untreated control.

45 Conclusions: The lead compounds DNDI-0690, DNDI-1047 and DNDI-6148 showed excellent activity  
46 across a range of *Leishmania* species *in vitro* and against *L. major* in mice. These compounds offer  
47 novel potential drugs for the treatment of CL.

48

49 **Keywords:** cutaneous leishmaniasis, drug discovery, aminopyrazole, benzoxaborole, nitroimidazole

50

51 **1 Introduction**

52 The leishmaniasis are a complex of diseases caused by *Leishmania* parasites with divergent disease  
53 manifestations, classified predominantly as visceral (VL) and cutaneous leishmaniasis (CL). There are  
54 over 15 species of *Leishmania* that cause different forms of CL, ranging from self-healing localised CL  
55 to chronic and disseminated CL, as well as mucosal leishmaniasis (MCL). As parasite transmission  
56 occurs via bites of the female sandfly, CL is often associated with skin lesions on exposed and visible  
57 areas of the body including the face. The disfigurement caused by this disease can result in  
58 psychological damage and stigma especially in women and children (Bennis et al., 2017; Kassi,  
59 Afghan, Rehman, & Kasi, 2008). However, as a non-fatal disease, CL remains one of the most  
60 neglected of neglected diseases in terms of drug discovery and development efforts.

61 Drugs and treatments used to cure CL today show many limitations (Aronson et al., 2017; Croft &  
62 Olliaro, 2011), which may be reflected in the absence of treatment-seeking behaviour of CL patients.  
63 All current treatments involve re-purposed drugs with a relatively high molecular weight and high  
64 polarity (except miltefosine) which results in poor oral bioavailability and limited room for  
65 optimisation of drug delivery through the skin (Bos & Meinardi, 2000; Hadgraft & Pugh, 1998;  
66 Lipinski, Lombardo, Dominy, & Feeney, 1997). There is a clear medical need for safe, effective and  
67 short-course treatment.

68 For successful treatment, a potent antileishmanial drug needs to reach the target site in the skin  
69 following either oral or topical drug administration to ensure effective treatment. The route of  
70 choice of drug administration is governed by both drug properties (Lipinski et al., 1997; Naik, Kalia, &  
71 Guy, 2000) and therapeutic concerns, and is an important factor in drug delivery.

72 Over the past decade, the Drugs for Neglected Disease *Initiative* (DNDi), a public private partnership  
73 that focusses on drug development for infectious diseases including neglected tropical diseases, has  
74 identified three highly potent anti-leishmanial chemical classes, the benzoxaboroles, the  
75 aminopyrazoles and the nitroimidazoles, with lead compounds for both VL and human African  
76 trypanosomiasis (HAT) (Jacobs, Plattner, Nare, et al., 2011; Mowbray et al., 2015; Thompson et al.,  
77 2018). Compounds from these series have shown (i) potent antileishmanial activity *in vitro*, (ii)  
78 favourable pharmacokinetic profiles to ensure bioavailability upon oral drug administration and (iii)  
79 high levels of activity against murine visceral leishmaniasis (Van den Kerkhof et al., 2018).

80 Nitroimidazoles are a class of anti-microbials that show broad spectrum activity against protozoans,  
81 mycobacteria and anaerobic bacteria. For example, metronidazole, a 5-nitroimidazole, is available in  
82 oral and vaginal dosage forms to treat *Trichomonas* and bacterial infections, and more specifically

83 the 2-nitroimidazole, benznidazole, is the front-line drug treatment for Chagas disease. Structural  
84 modifications have led to the discovery of two drugs, pretomanid (also known as PA-824, a 5-  
85 nitroimidazopyran) and delamanid (OPC-67683, a 6-nitro-2,3-dihydroimidazooxazole), for the  
86 treatment of multidrug-resistant tuberculosis caused by the intracellular *Mycobacterium*  
87 *tuberculosis* (Fairlamb & Patterson, 2018). Benzoxaboroles are bicyclic heterocycles in which the  
88 nitrogen of a benzo[c]isoxazole has been replaced by boron. Over the past decade, benzoxaboroles  
89 have been associated with a variety of antimicrobial properties that led to the initiation of several  
90 drug development programs. Tavaborole, another benzoxaborole, was approved by the FDA in 2014  
91 for the treatment of onychomycosis (Elewski & Tosti, 2014). Anacor Pharmaceuticals (now Pfizer),  
92 SCYNEXIS and DNDi investigated a wide range of antitrypanosomal benzoxaboroles analogues as  
93 part of a lead optimization program against HAT that resulted in the identification of acoziborole  
94 that is currently in Phase IIb/III trials (Jacobs, Plattner, & Keenan, 2011; Nare et al., 2010). AN13762  
95 is currently in preclinical development for malaria (Zhang et al., 2017) and DNDI-6148 is awaiting  
96 phase I clinical trials for VL. The antileishmanial activity of the aminopyrazoles was discovered  
97 through the high-throughput screening of a Pfizer small molecule diversity collection (C. E. Mowbray  
98 et al., 2015). In collaboration with Takeda Pharmaceutical Company Ltd, DNDi further optimised  
99 several potent aminopyrazole compounds for VL (Mowbray et al., 2015). Whilst DNDI-5561 was  
100 selected as the preclinical candidate, there are other promising candidates strengthening the  
101 pipeline of this class of compounds.

102 Here we report the evaluation of lead compounds of each of these three chemical classes aiming at  
103 the identification of a potential drug candidate to treat CL. It is important to note that the drug  
104 delivery targets for VL and CL are different - the liver, spleen and bone marrow, and the skin,  
105 respectively.

## 106 **2 Material and Methods**

107 *In vitro* and *in vivo* studies were conducted by two independent research groups at the London  
108 School of Hygiene & Tropical Medicine (LSHTM) and the Walter Reed Army Institute of Research  
109 (WRAIR) and methodologies are summarised in Table 1.

### 110 **2.1 Drugs and drug formulations**

111 LSHTM: Miltefosine was donated by Paladin Labs Inc and amphotericin B deoxycholate (Fungizone,  
112 E.R. Squibb & Sons, UK) was purchased from John Bell & Croyden Ltd. (London, UK). Both drugs were  
113 prepared to a stock concentration of 20mM in sterile PBS (0.9% NaOH, pH 7.4; Sigma Aldrich, UK)  
114 and sterile water respectively and stored at -20°C until required.

115 DNDi (Geneva, Switzerland) provided the experimental compounds, which were prepared as a stock  
116 solution of 20mM in dimethyl sulfoxide (DMSO, Sigma Aldrich, UK), sonicated (CamLab, Cambridge,  
117 UK) for 15 minutes and stored 4°C.

118 WRAIR: For the *in vitro* antileishmanial activity evaluation, the experimental compounds and  
119 solubilized amphotericin B (Fungizone, Sigma Aldrich, USA) were prepared at a stock solution of  
120 11.5mM/mg/mL in DMSO and stored at -20°C.

## 121 2.2 Parasite strains and animals

### 122 2.2.1 Parasite strains *in vitro* assays

123 LSHTM: *L. major* (MHOM/SA/85/JISH118), *L. mexicana* (MNYC/BZ/62/M379), *L. amazonensis* (*L.*  
124 *amazonensis*: DsRed2), *L. aethiopica* (MHOM/ET/84/KH) and *L. panamensis*  
125 (MHOM/PA/67/BOYNTON) amastigotes were isolated from mouse skin lesions. They were allowed  
126 to transform to promastigotes and were maintained in Schneider's insect medium (Sigma Aldrich,  
127 UK) (for *L. major*, *L. tropica* and *L. mexicana*) or M199 medium (Sigma-Aldrich, UK) (for *L.*  
128 *amazonensis*, *L. aethiopica* and *L. panamensis*) supplemented with 10% HiFBS at 26°C. *L. tropica*  
129 (MHOM/AF/2015/HTD7) was isolated from a skin biopsy of a CL patient that was inoculated into  
130 Novy-Nicolle-McNeil medium at the London Hospital of Tropical Diseases. Upon observation of  
131 parasite growth, the parasites were transferred to LSHTM, London, where the promastigotes were  
132 gradually adapted to Schneider's insect medium supplemented with 10% of HiFBS (Gibco, UK). Low  
133 passage number promastigotes (typically below passage number 3) were used for this experiment.

134 WRAIR: *L. major* (MHOM/IL/SU73/WR779), *L. guyanensis* (MHOM/GY/06/PAB-3985-WR-2853/A  
135 Chan), and *L. tropica* (MHOM/SU/74/K-27 WR-2995) were maintained in Schneider's insect medium  
136 (Lonza BioWhitaker, USA) supplemented with 20% heat inactivated fetal bovine serum (Corning,  
137 USA) at 22°C. All parasite lines were transfected with a luciferase-expressing construct as described  
138 in (Lecoeur et al., 2007).

### 139 2.2.2 Parasite strains for *in vivo* assays

140 LSHTM: All *Leishmania* strains were regularly passaged through mice to maintain virulence. Late  
141 stationary phase promastigote cultures were counted with a Neubauer hemocytometer using light  
142 microscopy (x40 magnification), centrifuged at 900 x *g* for 10 min at 4°C and re-suspended in RPMI  
143 medium without HiFBS to 2 x 10<sup>8</sup> promastigotes per mL. Mice were injected subcutaneously on the  
144 rump above the tail with 200µL of the promastigote suspension.

145 WRAIR: *L. major* promastigotes (NIH173 [MHOM/IR/-/173]) were harvested from infected BALB/c  
146 mouse footpads and were cultured in Schneider's medium (Lonza Life Sciences, Walkersville, MD)  
147 supplemented with 20% hiFBS. Cultures were maintained in T75 tissue culture flasks (Corning Life  
148 Sciences, Manassas, VA) at 22°C. Promastigotes for infection were harvested from the culture by  
149 spinning at 872 x *g* for 20 min. The medium was removed, and the resulting pellet was suspended in  
150 1× PBS. Two additional spins at 872 x *g* were conducted in PBS. After the second spin, a low volume  
151 of PBS was added, and stationary-phase promastigotes were counted and suspended at  $1 \times 10^8$   
152 parasites/mL. Animals were infected at the base of the tail with 100µL of parasite culture containing  
153  $1 \times 10^7$  *L. major* luciferase-expressing stationary-phase promastigotes.

#### 154 2.2.3 Animals and ethical statements

155 LSHTM: BALB/c mice (age 6-8 weeks) were purchased from Charles River (Margate, UK), whereas  
156 female CD-1 mice (age 7-8 weeks) were obtained in-house (London School of Hygiene & Tropical  
157 Medicine). The mice were housed in a controlled environment of 55% relative humidity and 26°C  
158 and provided with tap water and a standard laboratory diet. They were left to acclimatise for 5 days  
159 prior to the beginning of research studies.

160 All *in vivo* experiments were carried out under license (X20014A54) at the London School of Hygiene  
161 and Tropical Medicine (LSHTM) after discussion with the veterinarian and according to UK Home  
162 Office regulations.

163 WRAIR: Female BALB/c mice aged 6 weeks were purchased from Charles River Laboratories  
164 (Wilmington, MA). The mice were left to acclimatise for 7 days prior to the beginning of research  
165 studies. All mice were assigned a study number with an individual ear tag. All animals were  
166 quarantined for stabilization for 7 days prior to infection. Mice were housed in a designated room  
167 with food and water supplied ad libitum and a 12:12 light:dark cycle.

168 The animal protocol for this study was approved by the Walter Reed Army Institute of Research,  
169 Institutional Animal Care and Use Committee (Protocol number 16-ET-33) in accordance with  
170 national and Department of Defence guidelines. Research was conducted in an AAALACi accredited  
171 facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating  
172 to animals and experiments involving animals and adheres to principles stated in the Guide for the  
173 Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

174

175



176 2.3 *In vitro* antileishmanial activity

177 LSHTM: Peritoneal macrophages were isolated from CD-1 mice 24 hours after intraperitoneal starch  
178 induction. The macrophages were washed and re-suspended in RPMI-1640 with 10% heat-  
179 inactivated fetal bovine serum (HiFBS) at a density of  $4 \times 10^5$  macrophages per ml. Of this  
180 suspension, 100 $\mu$ L was added to each well of a 16-well Lab Tek slide and left to adhere for at 37°C in  
181 the presence of 5% CO<sub>2</sub>. After 24 hours, 100 $\mu$ L of stationary phase promastigotes of six different  
182 strains were counted, re-suspended in RPMI-1640 supplemented with 10% HiFBS and added in a  
183 ratio of 3:1 (*L. major* and *L. mexicana*), 5:1 (*L. tropica*, *L. panamensis* and *L. amazonensis*) and 7:1 (*L.*  
184 *aethiopica*) parasites to macrophages. The slides were left overnight at 34°C in a 5% CO<sub>2</sub>/ 95% air  
185 mixture.

186 Prior to adding drugs, the infection rate was evaluated. Briefly, the 24-hour control slide was fixed,  
187 stained with Giemsa and evaluated microscopically. In a minimum of four wells, 100 macrophages  
188 were evaluated for the presence or absence of amastigotes. If the result, expressed as percentage  
189 infection, was higher than 75% the experiment was continued. Stock solutions of the drugs in  
190 dimethylsulfoxide were prepared to a final concentration of 20mM and sonicated for 15 minutes.  
191 The cultures were washed to remove extracellular promastigotes and 100 $\mu$ L of the drug solution in  
192 RPMI-1640 supplemented with 10% HiFBS was added over a range of 30, 10, 3 and 1 $\mu$ M in  
193 quadruplicate for each concentration. Prior to adding the drug solutions to the infected  
194 macrophage cultures, the solubility of the test compound in RPMI-1640 with 10% HiFBS was  
195 evaluated using an inverted light microscope (x200). The presence of particles was evaluated as an  
196 indicator of solubility.

197 Amphotericin B deoxycholate (Fungizone®) and miltefosine were included as positive control drugs.  
198 After 72 hours of incubation at 34°C in a 5% CO<sub>2</sub>/ 95% air mixture, all slides were methanol-fixed  
199 and Giemsa-stained. The percentage inhibition was determined microscopically (x400  
200 magnification) as mentioned above. The EC<sub>50</sub> and EC<sub>90</sub> were calculated by non-linear sigmoidal  
201 curve fitting (variable slope) using Prism Software (GraphPad, Surrey, UK).

202 WRAIR: RAW 264.7 macrophages (ATCC, USA) were maintained in Dulbecco's modified eagle's  
203 medium (DMEM) (ATCC, USA) supplemented with 10% hiFBS (Corning, USA) at 37°C in an incubator  
204 supplied with 5% CO<sub>2</sub>. Macrophages were harvested from culture, assessed for viability using trypan  
205 blue, and re-suspended at  $2 \times 10^5$  cells/mL. The resulting suspension was dispensed at 50 $\mu$ L/well  
206 (10,000 macrophages/well) in 384-well white plates. After incubating for 24 hours at 37°C and 5%  
207 CO<sub>2</sub>, media was removed from the wells and replaced with 50 $\mu$ L of promastigote culture suspended  
208 in DMEM supplemented with 10% HiFBS (macrophage to promastigote ratio was 1:10 for *L. major*,

209 and 1:40 for *L. guyanensis* and *L. tropica*). Promastigotes were left to invade the macrophages for  
210 24 hours at 37°C and 5% CO<sub>2</sub>. Each well was washed three times in DMEM media supplemented  
211 with 10% hiFBS to remove any extracellular promastigotes and 77µL of drug solution in DMEM  
212 supplemented with 10% hiFBS was added over an initial testing range of 23000 to 10nM(2-fold  
213 serial dilutions across 12 wells) in quadruplicate for each concentration. Amphotericin B was  
214 included as a positive control and tested from 2160 to 1nM in octuplicate for each parasite strain.  
215 After 96 hours of incubation at 37°C and 5% CO<sub>2</sub>, Xenolight D-luciferin Potassium Salt (Perkin Elmer,  
216 USA) was added to each well at a final concentration of 150µg/mL and incubated for an additional  
217 30 minutes at 37°C and 5% CO<sub>2</sub>. Each plate was read for luminescence activity using an Infinite  
218 M200 plate reader (Tecan Inc., USA). EC<sub>50</sub>s were calculated for each drug using GraphPad Prism  
219 (GraphPad, USA) using the nonlinear regression (sigmoidal dose-response/variable slope) equation  
220 (Khraiweh et al., 2016).

#### 221 2.4 *In vitro* cytotoxicity (LSHTM)

222 KB cells were maintained in RPMI-1640 medium supplemented with L-glutamine and 10% HiFBS.  
223 This human-derived cell line was left in an incubator at 37°C and 5% CO<sub>2</sub> and passaged to new  
224 medium once a week (1/10 ratio). To assess cytotoxicity, the cells were counted and seeded in a 96-  
225 well plate at a concentration of 40,000 cells per well.

226 After a 24-hour incubation, the drug solutions were prepared by diluting the stock solution (20mM in  
227 DMSO) in RPMI-1640 with 10% HiFBS. The top concentration (200µM) was subsequently five-fold  
228 diluted across the plate. Podophyllotoxin was included as positive control drug (5µM highest test  
229 concentration). Untreated controls and blanks, containing only medium were also included. Each  
230 test compound was tested in triplicate. Plates were incubated for a further 72 hours at 37°C and 5%  
231 CO<sub>2</sub>.

232 After incubation, the wells were assessed microscopically and 20µl Alamar Blue<sup>®</sup> was added to each  
233 well. The plates were incubated for a further 2-4 hours before reading at EX/EM 560/585 (cut off  
234 570) in a Spectramax™ M3 Plate reader. The EC<sub>50</sub> value was calculated by non-linear sigmoidal curve  
235 fitting (variable slope) using Prism Software (GraphPad, Surrey, UK).

#### 236 2.5 Evaluation of physicochemical properties of drugs (LSHTM)

237 Physicochemical properties of the test compounds (partition coefficient (log D), H-bond donors and  
238 acceptors and molecular weight) were calculated using ChemDraw 3D 16.0 software (PerkinElmer,  
239 Waltham, UK).

240

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242

243 2.6 *In vivo* antileishmanial activity

244 2.6.1 Drug formulations

245 LSHTM: To maximise skin permeation for topical applications, saturated solutions in propylene  
246 glycol-ethanol (PG-EtOH 1:1) were prepared by adding an excess of drug compound to a glass vial  
247 together with 1mL of PG-EtOH (1:1) and a magnetic stirrer. The vial covered with aluminium foil was  
248 left at 34°C for 24 hours. An aliquot of this suspension was transferred to a vial and centrifuged for  
249 15 min at 18,407 x *g* and 34°C after which the supernatant was transferred to a clean vial and stored  
250 at 4°C until drug administration. For DNDI-VL-2098, a topical solution of 0.25mg/ml in PG-EtOH (1:1)  
251 was prepared. Drug concentrations and dosing frequency (for oral treatments) were determined  
252 based on efficacy observed against VL (Van den Kerkhof et al., 2018).

253 In preparation for the oral formulations, appropriate amounts of drugs were weighed and  
254 transferred to a clean glass vial. On the day of dosing, the exact volume of vehicle (Table 4) and glass  
255 beads were added to the vial before thoroughly mixing. The suspension was sonicated for 15 min  
256 prior to usage. Liposomal amphotericin B (AmBisome®, Gilead, UK) was prepared according to the  
257 manufacturer's instructions to a stock solution of 4mg/ml that was consequently diluted with 5%  
258 sterile dextrose (aq) to a 2.5mg/ml solution ready for use.

259 WRAIR: Nitroimidazole (DNDI-0690) was formulated in polyethylene glycol 400 (PEG400).  
260 Benzoxaborole (DNDI-6148) was formulated in 2% ethanol (EtOH), 1N sodium hydroxide (NaOH)  
261 (0.96 equiv), 5% dextrose (aq). Aminopyrazole (DNDI-1047) was formulated in 1% w/v  
262 methylcellulose (4000cps)/5% Tween 80/ddH<sub>2</sub>O. When needed, drugs were ground using a  
263 ProScientific 300D homogenizer and the particle size was measured using a Horiba LA-950V2 particle  
264 size analyser.

265 2.6.2 Murine CL model

266 LSHTM: The rump of female BALB/c mice were shaved using electric clippers (iClipper P6). Twenty-  
267 four hours later, stationary-phase *L. major* promastigotes were counted and re-suspended to a  
268 concentration of  $2 \times 10^8$  per ml in Schneider's insect medium. Each mouse was injected  
269 subcutaneously on the rump with 200µl of this parasite suspension. Approximately 10 days after  
270 infection a measurable nodule developed. The nodule diameter was calculated by averaging the

271 lesion diameters measured in 2 dimensions on a daily basis using digital callipers (Jencons Scientific  
272 Ltd., UK). When the nodule attained a diameter of approximately 4mm, the mice were re-grouped in  
273 groups of five to ensure similar nodule sizes in each group (one-way ANOVA,  $p > 0.05$ ) and drug  
274 treatment was started. Each experimental compound was administered both orally and topically. As  
275 a positive control either liposomal amphotericin B (25mg/kg/QAD; iv) or paromomycin sulfate  
276 (50mg/kg/day; ip) was included as well as a topical vehicle control to assess the impact of the vehicle  
277 alone.

278 The treatment efficacy was evaluated i) daily, by measuring the lesion size diameter and plotting as a  
279 function of time and ii) at the end of the experiment by quantification of the parasite load in the CL  
280 nodule by quantitative PCR (18S DNA target) at day 11 (one day after the last dose was  
281 administered) as described in detail by Van Bocxlaer et al (2018).

282 WRAIR: The lesion cure model was conducted as described in Caridha et al (2017a). The day before  
283 infection, the dorsolumbar regions (base of the tail) of the mice were shaved and hair was removed  
284 using NAIR™ to prevent quick hair re-growth. The shaved areas treated with NAIR were then washed  
285 with clean water 2-3 times and dried using clean gauze. On the day of infection each mouse was  
286 infected intra-dermally (ID) with 100 $\mu$ L parasite culture containing  $1 \times 10^7$  luciferase-expressing *L.*  
287 *major* stationary phase promastigotes. Starting from the third week post infection, the lesion  
288 induration diameters (length=D1 and width=D2) were measured using a calliper instrument (Fisher  
289 Scientific, USA) with 0.1 mm sensitivity. Length and width measurements were taken to account for  
290 asymmetrical lesions. Lesion size area was then calculated using the  $\pi R1 * R2$  formula (where  
291  $R1=D1/2$  and  $R2 = D2/2$ ). Lesions were measured at a 10-day (+/- 2 days) interval until the end of the  
292 study. Treatment was initiated approximately 3 -4 weeks post infections, when lesions progressed to  
293 an average size of approximately 20 mm<sup>2</sup>. Cohorts of five or six (respectively for study I and II)  
294 BALB/c mice were assigned to all treatment groups such that the mean lesion sizes for all groups  
295 were not statistically different from each other. The experimental endpoint for the murine cure  
296 model is lesion cure (100% re epithelialization or lesion size 0 x 0).

297 To measure the bioluminescence signal, luciferin (D-Luciferin potassium salt, Xenogen, CA and Gold  
298 Biotechnology, St. Louis, MO), the luciferase substrate, was inoculated intraperitoneally (IP) into  
299 BALB/c mice and glutathione at a concentration of 200mg/kg, 18 minutes before bioluminescence  
300 analysis. Animals were anaesthetized in a 2.5% isoflurane atmosphere (MWI Veterinary Supply,  
301 Harrisburg, PA) for 7 minutes and maintained in the imaging chamber for analysis. Emitted photons  
302 were collected by auto acquisition with a charge couple device (CCD) camera (PerkinElmer IVIS  
303 Spectrum In vivo Imaging System) using the medium resolution (medium binning) mode. Analysis  
304 was performed after defining a region of interest (ROI) that delimited the surface of the affected

305 area. Total photon emission from the base of the tail infected area was quantified with Living Image  
306 software (Xenogen Corporation, Alameda, CA), and results were expressed in numbers of  
307 photons/sec.

308 Experimental design: Two separate studies were conducted with the purpose of determining the  
309 efficacy of three compounds (nitroimidazole (DNDI-0690), benzoxaborole (DNDI-6148) and  
310 aminopyrazole (DNDI-1047)) in the BALB/c mouse/*L. major* lesion cure model.

311 In the first study, 10-day treatments of nitroimidazole (DNDI-0690) and aminopyrazole (DNDI-1047),  
312 as well as 5-day treatments of the benzoxaborole (DNDI-6148) compound, were administered orally  
313 (PO), twice a day (BID). Three vehicle control groups (2% EtOH, 5% dextrose (aq); PEG 400; and 0.5%  
314 w/v methylcellulose and 5% v/v Tween 80/ddH<sub>2</sub>O), were given once a day (QD), PO for 10  
315 consecutive days, except for 2% ETOH, 5% dextrose (aq) which was instead chosen to be given BID.

316 In the second study, the benzoxaborole (DNDI-6148) compound and the vehicle control group (2%  
317 ETOH, 5% dextrose (aq)) were administered PO, BID, for 10 consecutive days. In addition, in both  
318 studies, the positive control AmBisome<sup>®</sup> was administered intraperitoneally (IP), QD for 10  
319 consecutive days.

## 320 2.7 Statistical analyses

321 LSHTM: A one-way ANOVA with the Tukey post hoc test ( $p < 0.05$ , SPSSv23, IBM, Portsmouth, UK)  
322 was performed to indicate the statistical differences between average lesion diameters, surface  
323 area, and parasite loads of the group at the end of treatment. Further, repeated-measures ANOVA  
324 (Dunnett's Multi Comparison Test) ( $p < 0.05$ ) allowed the determination of whether the progression  
325 of lesion size in the experimental groups was statistically different from the included controls.

326 WRAIR: Statistical analysis was performed using the GraphPad Prism 7.04 software package  
327 (GraphPad Software, Inc., USA). One-way ANOVA with Dunnett's Multi Comparison Test and an  
328 unpaired t-test with Welch's correction were used to compare mean lesion size and  
329 bioluminescence signal differences between group means. A p-value  $< 0.05$  was considered  
330 statistically significant.

## 331 3 Results

### 332 3.1 *In vitro* antileishmanial activity

333 LSHTM: All experimental compounds consistently (within 10-fold range) showed potent activity  
334 against amastigotes in primary murine peritoneal macrophages for both New World (*L. mexicana*, *L.*  
335 *panamensis* and *L. amazonensis*) and Old World (*L. major*, *L. tropica* and *L. aethiopica*) species with

336 EC<sub>50</sub> values ranging from 0.22 to 24.61 μM (Table 2). Aminopyrazoles DNDI-1047, -1044 and -8012  
337 were the only compounds to demonstrate a nanomolar range activity against all *Leishmania* species  
338 in a similar range to amphotericin B. The *Leishmania* parasites are typically less susceptible to  
339 miltefosine, the other control drug, indicated by EC<sub>50</sub> values ranging from 9 to 36 μM (Escobar, Matu,  
340 Marques, & Croft, 2002; Van Bocxlaer et al., 2018). Together with the above mentioned  
341 aminopyrazoles, the benzoxaborole and the nitroimidazoles demonstrated consistently high activity  
342 (EC<sub>50</sub> < 5 μM) against an Old and a New World strain and occasionally lower levels of activity (5 μM  
343 < EC<sub>50</sub> < 25 μM) against one or two specific strains.

344 WRAIR: Experimental results are shown in Table 3. As with the *in vitro* testing conducted against  
345 peritoneal macrophages, all experimental compounds consistently (within 10-fold) demonstrated  
346 nM range activity against both Old World (*L. major* and *L. tropica*) and New World (*L. guyanensis*)  
347 species when tested in the assay using amastigotes in a macrophage cell line assay. All tested drugs  
348 demonstrated *in vitro* efficacies similar to amphotericin B.

### 349 3.2 Cytotoxicity

350 Cytotoxicity assessment of the compounds demonstrated at least 10-fold selectivity between  
351 *Leishmania* species and host cells for all test compounds. The EC<sub>50</sub> values of the test compounds  
352 when incubated with KB cells were above the highest test concentration (200 μM), except for DNDI-  
353 1044 (aminopyrazole) and DNDI-6148 (benzoxaborole) with EC<sub>50</sub> values of 48.89 μM and 180.70 μM,  
354 respectively.

### 355 3.3 Physicochemical property evaluation

356 All tested candidates have physicochemical properties (Table S1) consistent or approaching values  
357 recommended as suitable to allow passive skin permeation except for the partition (for non-  
358 ionisable molecules) or distribution (for ionisable molecules) coefficient and number of H-bond  
359 acceptors in the case of DNDI-0690 and DNDI-6148. With a distribution coefficient (log D) of 1.92  
360 (pH 7.4, DNDi unpublished data) the benzoxaborole (DNDI-6148) is more hydrophilic than the  
361 lipophilic nitroimidazole (DNDI-0690 – log D (pH 7.4) = 2.45, DNDi unpublished data) and  
362 aminopyrazole (DNDI-1047 – log D (pH 7.4) = 3.68, DNDi unpublished data). All three are within or  
363 only just borderline outside the ideal skin permeant range (1 < log D < 3, (Hadgraft & Pugh, 1998))  
364 and it was, therefore, decided to further evaluate their therapeutic potential against experimental  
365 CL.

### 366 3.4 Drug activity against murine CL

367 LSHTM: The antileishmanial activities for each drug and dose regimen, expressed as both parasite  
368 load and lesion size reduction compared to the appropriate control groups, are shown in Table 5 and  
369 illustrated in Figure 1. Even though different regimens were evaluated, it is clear that DNDI-0690, -  
370 6148 and -1047 were able to significantly reduce the lesion size and parasite load in mice upon oral  
371 administration. At an equivalent dose of 25mg/kg/day (i.e. 12.5 mg/kg/day BID or 25 mg/kg/day  
372 QD), DNDI-1047 was most effective in reducing the lesion size at the end of treatment, followed by  
373 DNDI-0690 and DNDI-6148 with 100%, 86.4% and 66.7% reduction, respectively. The reduction of  
374 parasite loads in the skin follows a similar trend, suggesting an appropriate distribution of the  
375 compounds to the location of the parasite. A linear correlation between the administered dose and  
376 the lesion size and parasite reduction was observed for DNDI-0690, DNDI-1047 and DNDI-6148  
377 (Table 5).

378 When comparing the efficacy of the different drug compounds applied as saturated solution in a  
379 propylene glycol-ethanol (1:1, v:v) upon topical administration, the benzoxaborole (DNDI-6148) and  
380 the aminopyrazoles (DNDI-1044, -1047 and -8012) were found to reduce both the parasite load and  
381 the lesion size, suggesting permeation of the compound into the dermis. The nitroimidazoles, DNDI-  
382 0690 and DNDI-VL-2098, were the only compounds unable to significantly reduce the lesion size  
383 (23.6% and 23.5% reduction compared to the topical vehicle only control group, respectively). All  
384 treatments were well-tolerated and no overt signs of toxicity were observed in the mice.

385 WRAIR: Experimental results from the first *in vivo* study are shown in Figure 2. In this study, the  
386 lesion sizes in all study groups were measured on days 3, 11, 20, 28 and 34 days post end of  
387 treatment.

388 After treatment ended, the lesion sizes in the benzoxaborole (DNDI-6148) and nitroimidazole (DNDI-  
389 0690) treated groups started decreasing at a similar rate to that of the AmBisome® treated group.  
390 On day 11 post end of treatment, 5/5 BALB/c and 1/5 BALB/c mice were clear of any detectable  
391 infection in the AmBisome® and benzoxaborole treated groups respectively, despite the fact that  
392 benzoxaborole treated animals received only 5 days' worth of treatment compared to the 10 day  
393 treatments for all the other groups. Overall, when compared to their respective vehicle control  
394 groups, lesion sizes on day 11 post end of treatment were significantly reduced by 93.2%, 89.8%,  
395 73.8% and 100% respectively for the benzoxaborole (DNDI-6148), nitroimidazole (DNDI-0690),  
396 aminopyrazole (DNDI-1047) and AmBisome® groups. Lesion sizes in the three vehicle control (VC)  
397 groups were not statistically different from each other. After day 11 post end of treatment, with the  
398 exception of the AmBisome® treated group, lesion sizes continued to increase as shown in Figure 2.

399 Bioluminescence signal progression in all treatment groups is shown in Figure 3. On the day post end  
400 of treatment all groups including the AmBisome<sup>®</sup>, nitroimidazole, aminopyrazole and the  
401 benzoxaborole displayed a statistically significant reduction of parasite load when compared with  
402 their respective VC. The bioluminescence signal exponentially increased in all groups except for the  
403 AmBisome<sup>®</sup> treated group either immediately (DNDI-1047 and DNDI-0690) or a few days (DNDI-  
404 6148) after treatment ended. Both readouts (i.e. lesion size and *in vivo* bioluminescence) seem,  
405 therefore, to correlate well to post-treatment outcome of the monitored treated groups.

406 On day 28 post end of treatment all vehicle control groups, as well as the aminopyrazole (DNDI-  
407 1047) group, were euthanized due to large lesion sizes. Animal groups treated with either the  
408 benzoxaborole (DNDI-6148) or the nitroimidazole (DNDI-0690) were monitored until day 34 post end  
409 of treatment, on which day they were euthanized due to rapidly increasing lesion sizes. On this day  
410 one BALB/c mouse belonging to the AmBisome<sup>®</sup> group had relapsed.

411 Experimental results from the second lesion cure study are shown in Figure 4. Lesion sizes were  
412 measured on days 4, 11, 18, 25, 34, 39, 44, 53, 61, 66, 74 and 80 post end of treatment. After  
413 treatment ended, the lesion sizes in the benzoxaborole (DNDI-6148) treated group started  
414 decreasing at a much faster rate compared to the AmBisome treated group. On day 11 post end of  
415 treatment 2/6 and 3/6 BALB/c mice belonging to the AmBisome<sup>®</sup> and benzoxaborole (DNDI-6148)  
416 treated groups respectively were cured (no visible lesion) and did not present any sign of detectable  
417 parasite load as measured by bioluminescence. The overall lesion sizes were reduced by 78.8% and  
418 99.3% respectively compared to the average VC lesion size. On day 18 post end of treatment 5/6 and  
419 6/6 BALB/c mice belonging to the AmBisome<sup>®</sup> and benzoxaborole (DNDI-6148) treated groups  
420 respectively were cured and did not show any sign of detectable parasite load. On day 25 post end  
421 of treatments all animals of both treatment groups showed no visible lesions whereas the VC group  
422 was euthanized because of big lesion sizes.

423 On day 46 post end of treatment, the bioluminescence signal was assessed again with the purpose  
424 of determining possible relapses of the *L. major* infection at the inoculation site and evaluating the  
425 parasite load in both treatment groups. Parasites were visible in 2/5 and 4/6 BALB/c mice that  
426 belonged to the benzoxaborole (DNDI-6148) and AmBisome<sup>®</sup> treated groups respectively (Figure  
427 S1). The overall parasite load evaluated by the intensity of the bioluminescence signal in the  
428 AmBisome<sup>®</sup> group was 32% higher than the parasite load in the benzoxaborole (DNDI-6148) treated  
429 group.

430 On day 61 post end of treatment, 3/6 and 0/5 BALB/c mice belonging to the AmBisome<sup>®</sup> and  
431 benzoxaborole (DNDI-6148) treated groups respectively had developed papules and onn day 74 post



432 end of treatment, this had increased to 4/6 and 1/5 mice, respectively. On day 80, which was the last  
433 day of the study, 4/6 and 1/5 mice belonging to the AmBisome® and benzoxaborole treated groups  
434 had developed lesions with average sizes of 11.2 mm<sup>2</sup> and 1.98 mm<sup>2</sup> respectively; these values were  
435 statistically different from each other.

436 An unpaired t-test with Welch's correction, which was used to compare bioluminescence signal  
437 differences between group means on day 46 post end of treatment, showed no statistically  
438 significant difference between the two treated groups at this time point. Despite that, there seems  
439 to be a good correlation between the reappearance (relapse) of the *L. major* infections at the former  
440 lesion site and the formation of lesions at the same site within 1-2 weeks. The lack of statistical  
441 significance in the overall parasite load difference for the AmBisome® and the benzoxaborole (DNDI-  
442 6148) treated groups was probably due to the high variability in the bioluminescence signal emitted  
443 from animals belonging to the same treatment group.

444 In previous studies we have shown that, the bioluminescence signal (which reflects the parasite  
445 load) emitted by the infection sites in BALB/c mice infected with luciferase-expressing *L. major*  
446 parasites reaches a plateau and/or begins to diminish at approximately 35 days post infection. This is  
447 probably due to signal attenuation associated with the appearance of dark crust on lesions and not  
448 to an intrinsic decrease of the *in vivo* signal (Caridha et al., 2017b). For this reason, bioluminescence  
449 signal data observed for each animal was collected and followed as a means of evaluating the  
450 parasite load at the infection site but was not used as a main experimental endpoint in this study.

451

#### 452 **4 Conclusion**

453 One of the major drug discovery challenges for CL is to find a drug that has i) a potent activity against  
454 the many different causative species and ii) the properties to ensure therapeutic drug exposure in  
455 the skin. To address this first need, both New World and Old World CL species were included in an *in*  
456 *vitro* drug susceptibility evaluation panel. All drug candidates tested demonstrated marked to  
457 outstanding levels of potency against Old and New World species regardless of the Institute and/or  
458 evaluation assay used. When tested in the *in vitro* peritoneal assay (LSHTM), the aminopyrazoles  
459 showed the most potent antileishmanial activity of the evaluated compounds with nanomolar-range  
460 EC<sub>50</sub> values similar to amphotericin B. At WRAIR, all compounds demonstrated *in vitro* efficacies with  
461 nanomolar-range EC<sub>50</sub> values similar to amphotericin B against both Old World (*L. major* and *L.*  
462 *tropica*) and New World (*L. guyanensis*) species.

463 The currently available drugs (apart from miltefosine), amphotericin B, paromomycin, and the  
464 pentavalent antimonials are high MW polar molecules, which accounts for their poor oral

465 bioavailability and their need to be injected or infused. It is therefore an important part of the  
466 approach by DNDi and others to focus on bioavailability early on in the drug discovery process,  
467 resulting in the design of molecules with the drug-like properties needed for them to reach the  
468 required target sites (C E Mowbray, 2018), including their physicochemical properties (C. E.  
469 Mowbray et al., 2015; Mukkavilli et al., 2014; Thompson et al., 2017; Van Bocxlaer et al., 2018). This  
470 approach was clearly justified by the significant efficacy demonstrated by the potent lead  
471 compounds when delivered orally or topically (except as explained below for the nitroimidazoles) in  
472 mouse models of infection. In two independent mouse models of CL infection, compounds of all  
473 three classes demonstrated antileishmanial activity following oral administration indicated by  
474 significant – and in some cases complete - lesion size reduction, which correlated with a reduction in  
475 parasite load determined by both quantitative PCR and bioluminescent signal in the experimental  
476 treatment groups when compared to untreated and/or vehicle controls. The efficacy against  
477 experimental CL was both dose- and treatment duration-dependent in the different models used at  
478 LSHTM and WRAIR respectively.

479 When applied to the skin topically as a saturated solution, the benzoxaborole (DNDI-6148) and the  
480 aminopyrazoles (DNDI-1047, DNDI-1044 and DNDI-8012) were able to significantly reduce lesion size  
481 and some even reduced the parasite load in the skin. Given the complex architecture of the skin,  
482 stricter thresholds of physicochemical parameters are imposed for topical drug delivery (Choy &  
483 Prausnitz, 2011). This was apparent for the poorly soluble nitroimidazole DNDI-VL-2098, which was  
484 unable to reduce the lesion size when applied locally to the skin. This could be explained by the  
485 lower diffusive driving force of the DNDI-VL-2098 topical formulation that only contained 0.25mg/ml  
486 of DNDI-VL-2098 (diffusive driving force < 1), whereas the other molecules were applied as a  
487 saturated solution (diffusive driving force of 1). The second nitroimidazole DNDI-0690 is also poorly  
488 soluble probably contributing to the limited activity when administered topically. CL pathology  
489 resulting from parasites residing in the skin may also influence topical drug delivery, for example due  
490 to the induced hydrophilic environment in the dermis (Van Bocxlaer, Yardley, Murdan, & Croft,  
491 2016); CL pathology has been shown to have an impact upon systemic drug delivery (Wijnant, Van  
492 Bocxlaer, Yardley, Harris, Alavijeh, et al., 2018; Wijnant, Van Bocxlaer, Yardley, Harris, Murdan, et al.,  
493 2018).

494 Only a handful of known antileishmanial compounds (AmBisome® and paromomycin being two of  
495 these) have established efficacy and can cure lesions in these stringent models of rodent CL. The  
496 superior activity demonstrated by all three lead compounds, in two independent laboratories (see  
497 Table 1 for comparison of methods), is a strong testimony of the high antileishmanial efficacy of  
498 these three lead compounds (Caridha et al., 2017a).

499 CL affects poor people, often living in rural and remote areas that only seek treatment  
500 approximately 1 to 6 months after the first symptoms (Ruoti et al., 2013), when the disease has  
501 already progressed to a stage where scars can no longer be avoided. It is hence of great importance  
502 to select drugs that fit the target product profile which involves a i) safe ii) short course iii) patient-  
503 friendly iv) oral and/or topical treatment with v) stability in tropical climates in order to encourage  
504 early treatment-seeking behaviour. DNDI-0690, DNDI-6148 and DNDI-5561 have all been nominated  
505 as preclinical candidates for VL and the first two of these are already scheduled to progress into  
506 clinical development in the near future (Croft, Chatelain, & Barrett, 2017). Even though the target  
507 tissue for CL, the skin, is different to that for VL, where the drugs should reach therapeutic  
508 concentrations in the liver and spleen, the results reported here show that these compounds also  
509 represent promising classes for the therapy of CL.

510

#### 511 **Acknowledgements**

512 DNDi received financial support for this work from the following donors: Federal Ministry of  
513 Education and Research (BMBF) through KfW, Germany; Dutch Ministry of Foreign Affairs (DGIS), the  
514 Netherlands; World Health Organization – Special Programme for Research and Training in Tropical  
515 Diseases (WHO-TDR); and for its overall mission from UK aid, UK; Médecins sans Frontières (MSF)  
516 and the Swiss Agency for Development and Cooperation (SDC), Switzerland.

#### 517 **Disclaimer**

518 Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to  
519 its presentation and/or publication. The opinions or assertions contained herein are the private  
520 views of the author, and are not to be construed as official, or as reflecting true views of the  
521 Department of the Army or the Department of Defense.  
522 Research was conducted under an approved animal use protocol in an AAALACi accredited facility in  
523 compliance with the Animal Welfare Act and other federal statutes and regulations relating to  
524 animals and experiments involving animals and adheres to principles stated in the Guide for the Care  
525 and Use of Laboratory Animals, NRC Publication, 2011 edition.

526

527 **Tables.**528 **Table 1.** Comparison of the *In vitro* and *in vivo* assay design at LSHTM and WRAIR.

	LSHTM	WRAIR
<b><i>In vitro</i> assay</b>		
<b>Host cells</b>	peritoneal macrophages from CD-1 mice	RAW 264.7
<b><i>Leishmania</i> strain</b>	<i>L. major</i> (MHOM/SA/85/JISH118) <i>L. mexicana</i> (MNYC/BZ/62/M379) <i>L. amazonensis</i> ( <i>L. amazonensis</i> : DsRed2) <i>L. aethiopica</i> (MHOM/ET/84/KH) <i>L. panamensis</i> (MHOM/PA/67/BOYNTON)	<i>L. major</i> (MHOM/IL/SU73/WR779) <i>L. guyanensis</i> (MHOM/GY/06/PAB-3985-WR-2853/A Chan) <i>L. tropica</i> (MHOM/SU/74/K-27 WR-2995)
<b>Assay medium</b>	RPMI-1640 + 10%HiFBS	DMEM + 10%HiFBS
<b>Assay format</b>	16 –well Lab Tek slide	384-well plate
<b>Drug start concentration</b>	10uM – 1:3 dilutions	10ug/ml – 1:2 dilutions
<b>Drug incubation time</b>	72h	96h
<b>Assay temperature</b>	34°C from infection onwards	37°C from infection onwards
<b>Drug solutions</b>	100% DMSO – in assay: < 1% DMSO	100% DMSO – in assay: 0.2% DMSO
<b>Control drugs</b>	Amphotericin B (Fungizone) and miltefosine	Amphotericin B
<b>Read out</b>	Microscopic counting Ratio of infected cells upon drug treatment vs untreated controls	Bioluminescent signal Total parasite counting (signal based)
<b><i>In vivo</i> assay</b>		
<b><i>Leishmania</i> strain</b>	<i>L. major</i> (MHOM/SA/85/JISH118)	<i>L. major</i> (NIH173 (MHOM/IR/-/173)
<b>Mouse strain</b>	Female BALB/c mice – 6-8 weeks old (Charles River)	Female BALB/c mice – 6 weeks old (Charles River)
<b>Group size</b>	5	5-6
<b>Infection</b>	Low passage (p<5) stationary phase promastigotes	Stationary phase promastigotes
<b>Inoculum size</b>	200ul containing 4x10 <sup>7</sup> promastigotes	100ul containing 1x10 <sup>7</sup> promastigotes
<b>Place of infection</b>	Rump above the tail	Rump above the tail
<b>Treatment initiation</b>	Average nodule diameter of 3-4mm	Average nodule surface of 20mm <sup>2</sup>
<b>Drug efficacy assessment</b>	Reduction vs untreated or vehicle control of: 1) Daily lesion size measurements 2) Parasite load (qPCR) at end of treatment	Reduction vs untreated or vehicle control of: 1) Lesion size measurements 2) Bioluminescence signal
<b>Study duration</b>	11 days after the first dose administration	Until relapse occurs
<b>Positive control</b>	AmBisome®, IV, 25 mg/kg QAD, 10 days Paromomycin, IP, 50mg/kg, QD, 10 days	AmBisome®, IP, 25 mg/kg QD, 10 days

529

530

531

532 **Table 2.** Susceptibility of a range of *Leishmania* species that cause CL against the experimental compounds ( $EC_{50}$  in  $\mu M$  (95% confidence interval),  $n$  is experiment number)  
 533 [LSHTM data]

	Compound ID	n	<i>L. major</i>	<i>L. tropica</i>	<i>L. aethiopia</i>	<i>L. mexicana</i>	<i>L. panamensis</i>	<i>L. amazonensis</i>	Cytotoxicity
Reference drugs	Amphotericin B	1	0.07 (0.06 – 0.09)	0.07 (0.07–0.08)	0.11 (0.10 – 0.13)	0.78 (1.6 – 0.20)	0.07 (0.07 – 0.08)		
		2	0.03 (0.03 – 0.03)	0.34 (0.25 – 0.46)		0.08 (0.06 – 0.19)		0.13 (0.09–0.20)	
	Miltefosine	1	28.89	35.20	36.10 (27.95 – 46.64)	11.21 (7.57 – 16.60)	21.33 (18.91 – 24.06)		
		2	30.02	29.52		9.22 (7.35 – 11.56)		14.95 (12.08 – 18.49)	> 200
Nitroimidazoles	DNDI-0690	1	4.56 (2.73 – 7.62)	1.41 (1.29 – 1.53)	24.61	1.91 (1.60 – 2.31)	0.77	< 1.11	< 1.11
		2	7.94 (4.31 – 14.64)	2.38 (1.91 – 2.97)		< 1.11		< 1.11	> 200
	DNDI-VL-2098	1	0.83 (0.62 – 1.12)	1.34 (1.17 – 1.54)		5.75 – 7.73)			> 200
		1	3.24 (1.09 – 9.69)	1.33 (0.95 – 1.85)	3.17 (2.84 – 3.53)	1.17 (0.82 – 1.68)	0.34 (0.31 – 0.37)	4.68	> 200
Benzoxaboroles	DNDI-8219	2	1.44 (1.30 – 1.60)	1.80 (1.66 – 1.96)		< 1.11		4.68 (3.57 – 6.12)	
		1	2.10 (1.70 – 2.60)	7.25 (6.00 – 8.77)	12.35 (8.51 – 17.92)	2.36 (1.79 – 3.12)	18.26 (9.58 – 34.80)	2.04	180.70
	DNDI-6148	2	1.20 (0.61 – 2.33)	6.54 (4.17 – 10.25)		< 1.11		< 1.11	
		1	0.63 (0.56 – 0.72)	0.83 (0.78 – 0.89)	0.29 (0.28 – 0.32)	< 0.33	< 0.33	< 1.11	48.89
Aminopyrazoles	DNDI-1044	2	< 1.11	< 1.11		< 1.11		< 1.11	
		1	0.24 (0.22 – 0.26)	0.34 (0.31 – 0.37)	< 0.33	< 0.33	< 0.33	< 1.11	> 200
	DNDI-1047	2	< 1.11	< 1.11		< 1.11		< 1.11	
		1	0.62 (0.58 – 0.67)	0.71 (0.61 – 0.84)	0.39 (0.38 – 0.41)	< 0.33	< 0.33	< 1.11	> 200
	DNDI-8012	2	< 1.11	< 1.11		< 1.11		< 1.11	

534

535 **Table 3.** Susceptibility of a range of *Leishmania* species in the luciferase amastigote macrophage assay that cause CL against the experimental compounds ( $EC_{50}$  in  $\mu M$  (95%  
 536 confidence interval),  $n$  is number of experiments) [WRAIR data]

	Compound ID	n	<i>L. major</i>	<i>L. guyanensis</i>	<i>L. tropica</i>
Reference Drug	Amphotericin B	2	0.03 (0.004–0.014)	0.03 (0.003 – 0.013)	0.01 (0.001 – 0.002)
Nitroimidazoles	DNDI-0690	2	0.34 (0.27 – 0.39)	0.85 (0.18 – 2.61)	2.36 (1.69 – 2.18)
	DNDI-8219	2	0.03 (0.02 – 0.03)	0.38 (0.05 – 2.14)	1.20 (1.63 – 2.24)
Benzoxaboroles	DNDI-6148	2	0.07 (0.05 – 0.11)	0.05 (0.05–0.06)	0.24 (0.16 – 0.31)
	DNDI-1044	2	0.02 (0.02 – 0.03)	0.03 (0.02 – 0.03)	0.05 (0.02 – 0.05)
Aminopyrazoles	DNDI-1047	2	0.01 (0.01 – 0.02)	0.02 (0.01 – 0.03)	0.06 (0.03 – 0.10)
	DNDI-8012	2	0.02 (0.01 – 0.03)	0.03 (0.03 – 0.04)	0.07 (0.04 – 0.08)

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**Table 4.** *In vivo* study design including dose regimen and formulation details.

Group	Active compound	Vehicle	Administration route	Treatment regimen
Untreated control	N/A	N/A	N/A	N/A
Liposomal amphotericin B (AmBisome®)	Amphotericin B	Dextrose 5%	Intravenous	25mg/kg (2.5mg/ml) QAD, 5 doses
Paromomycin	Paromomycin sulfate	PBS	Intraperitoneal	50 mg/kg (5mg/ml) QD, 10 days
Vehicle control topical	N/A	PG-EtOH (1:1)	Topical	50ul BID, 10 days
Experimental topical formulation 1	Aminopyrazoles Benzoxaboroles Nitroimidazoles	PG-EtOH (1:1)	Topical	50µl of saturated drug solution BID, 10 days
Experimental oral formulation 1	Nitroimidazoles: DNDI-0690 DNDI-VL-2098	Polyethylene glycol 400 10% of Tween 80- EtOH (7:3) in ddH <sub>2</sub> O	Oral	6.25mg/kg (0.625mg/ml), 12.5mg/kg (1.25mg/ml), 25mg/kg (2.5mg/ml) or 50mg/kg (5.0mg/ml) QD, 10 days
Experimental oral formulation 2	Aminopyrazoles	1% methylcellulose (w/v, 4000cps)/5% Tween 80/ddH <sub>2</sub> O	Oral	3.125mg/kg (0.3125mg/ml), 6.25mg/kg (0.625mg/ml), 12.5mg/kg (1.25mg/ml), 25mg/kg (2.5mg/ml) or 50mg/kg (5.0mg/ml) BID, 5 or 10 days
Experimental oral formulation 3	Benzoxaboroles	2% ethanol, NaOH (1M), 5% dextrose	Oral	12.5mg/kg (1.25mg/ml), 25mg/kg (2.5mg/ml) or 50mg/kg (5.0mg/ml) BID or QD, 5 or 10 days

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553 **Table 5.** The efficacy as mean % reduction of parasite load and lesion size of the lead nitroimidazoles,  
 554 benzoxaboroles and aminopyrazoles upon treatment with different dosing regimens (n=5).

	Compound	Administration route	Dose (mg/kg)	Application frequency	Duration (days)	% reduction	
						Lesion size	Parasite load
Reference	Liposomal amphotericin B	IV	25	QAD	10	59.1	95.14
Nitroimidazole	DNDI-0690	Oral	6.25	QD	10	72.1	50.19
		Oral	12.5	QD	10	74.3	81.24
		Oral	25	QD	10	86.4	84.18
		Oral	50	QD	10	100.0	94.89
		Topical	Sat sol	BID	10	23.6	50.74
	DNDI-VL-2098	Oral	12.5	QD	10	45.0	62.68
		Oral	25	QD	10	95.4	98.50
		Topical	0.25mg/ml	BID	10	23.5	49.40
Benzoxaborole	DNDI-6148	Oral	12.5	BID	10	66.7	92.35
		Oral	25	BID	10	85.4	98.49
		Oral	50	BID	5	43.2	100.00
		Oral	50	QD	10	100.0	97.73
		Oral	50	BID	10	100.0	99.56
		Oral	50	BID	10	75.7	100.00
		Oral	50	BID	10	91.9	98.41
		Topical	Sat sol	BID	10	71.4	71.49
		Topical	Sat sol	BID	10	91.3	99.68
		Aminopyrazole	DNDI-1044	Oral	25	BID	10
Oral	25			BID	10	100.0	99.96
Topical	Sat sol			BID	10	100.0	99.59
DNDI-1047	Oral		3.125	BID	10	17.0	64.32
	Oral		6.25	BID	10	56.7	94.65
	Oral		12.5	BID	10	100.0	99.96
	Oral		12.5	BID	10	100.0	97.17
	Oral		25	BID	10	100.0	99.63
	Oral		25	BID	10	83.2	99.41
	Oral		50	BID	5	66.1	97.97
	Topical		Sat sol	BID	10	100.0	98.22
DNDI-8012	Oral		25	BID	10	92.0	99.96
	Oral		25	BID	10	86.8	99.38
	Topical		Sat sol	BID	10	93.8	100.00

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557 **Figure Legends**

558 **Figure 1. Efficacy of the nitroimidazole (DNDI-0690), benzoxaborole (DNDI-6148) and**  
559 **aminopyrazole (DNDI-1047) in the *L. major*-BALB/c model of CL.** Mice received 25 mg/kg of  
560 liposomal amphotericin B (IV) every other day or 50mg/kg of paromomycin sulfate (IP) once daily or  
561 50mg/kg of DNDI-0690 once daily (oral) or 25mg/kg of DNDI-1047 or DNDI-6148 (oral) twice daily.  
562 For topical treatment, 50µl of a saturated solution in PG-EtOH was applied twice daily. All  
563 treatments were continued for 10 days. During treatment, lesion size was measured daily (a). The  
564 average lesion diameter represents the mean (n = 5). On day 11, lesion skin samples were collected  
565 and parasite load (b) was quantified. Each marker represents 1 parasite load. One-way ANOVA for  
566 parasite load and repeated measures for lesion size followed by Tukey's multiple comparison tests  
567 was used to analyse differences between untreated controls and experimental groups. A p-value <  
568 0.05 was considered statistically significant.

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570 **Figure 2. Nitroimidazole (DNDI-0690), benzoxaborole DNDI-6148), and aminopyrazole (DNDI-1047)**  
571 **efficacy in the lesion cure model in BALB/c mice infected with luciferase-expressing *L. major***  
572 **parasites.** Mice received 50 mg/kg DNDI-6148, 25 mg/kg DNDI-0690, and 25 mg/kg DNDI-1047. All  
573 drugs were given PO, BID, for 10 consecutive days except for DNDI-6148 which was given for 5  
574 consecutive days. The positive control group was treated with 25 mg/kg AmBisome® which was  
575 given IP, QD for 10 consecutive days. Three vehicle control groups VC 1, VC 2, and VC 3 consisted of  
576 2% ETOH, 5% dextrose (aq); PEG 400; and 0.5% w/v methylcellulose, 5% v/v Tween 80/ddH<sub>2</sub>O  
577 respectively, which were the solvents used to dissolve DNDI-6148, DNDI-0690, and DNDI-1047  
578 respectively. All vehicle control groups were treated PO, QD for 10 consecutive days, except for the  
579 VC1 which was randomly chosen to be administered BID. The average lesion size represents the  
580 mean ± standard error for each time point. One-way ANOVA was used to analyse differences  
581 between the positive, negative, and experimental groups. A p-value < 0.05 was considered  
582 statistically significant (\*: p < 0.05).

583 **Figure 3. Nitroimidazole (DNDI-0690), benzoxaborole (DNDI-6148), and aminopyrazole (DNDI-**  
584 **1047) significantly reduce the bioluminescence signal (parasite load) at the infection site in BALB/c**  
585 **mice infected with luciferase-expressing *L. major* parasites.** Mice received 50 mg/kg DNDI-6148, 25  
586 mg/kg DNDI-0690, and 25 mg/kg DNDI-1047. All drugs were given PO, BID, for 10 consecutive days  
587 except for DNDI-6148 which was given for 5 consecutive days. The positive control group was  
588 treated with 25 mg/kg AmBisome® which was given IP, QD for 10 consecutive days. Vehicle control  
589 groups (VC 1, VC 2, and VC 3) consisted of 2% ETOH, 5% dextrose (aq); PEG 400; and 0.5% w/v  
590 methylcellulose, 5% v/v Tween 80/ddH<sub>2</sub>O respectively, which were the solvents used to dissolve  
591 DNDI-6148, DNDI-0690, and DNDI-1047 respectively. All vehicle control groups were treated PO, QD  
592 for 10 consecutive days, except for the VC1, which was chosen to be administered BID instead of QD.  
593 Each point represents mean ± standard error for the bioluminescence signal. One-way ANOVA was  
594 used to analyze differences between the positive, negative, and experimental groups. A p-value <  
595 0.05 was considered statistically significant (\*: p < 0.05).

596 **Figure 4. Benzoxaborole (DNDI-6148) efficacy in the lesion cure model in BALB/c mice infected**  
597 **with luciferase-expressing *L. major* parasites.** Mice received 50 mg/kg DNDI-6148 PO, BID, for 10  
598 consecutive days. The positive control group received 25 mg/kg AmBisome® IP, QD for 10  
599 consecutive days. The vehicle control group was treated PO, BID with 2% ETOH, 5% dextrose (aq).  
600 DNDI-6148 was formulated in 2% ETOH, 1N NaOH (0.96 equiv), 5% dextrose (aq). The average lesion  
601 size represents the mean + standard error for each time point. An unpaired t-test with Welch's



602 correction were used to compare mean lesion size and bioluminescence signal differences between  
603 group means. A p-value < 0.05 was considered statistically significant (\*: p < 0.05).

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