

1 **Structural requirements for dihydrobenzoxazepinone**
2 **anthelmintics: actions against medically important and model**
3 **parasites - *Trichuris muris*, *Brugia malayi*, *Heligmosomoides***
4 ***polygyrus* and *Schistosoma mansoni***

5

6 Short title: (70 characters) Dihydrobenzoxazepinone efficacy on human and model helminth parasites

7

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37

38 **Abstract**

39 Nine hundred million people are infected with the soil-transmitted helminths *Ascaris lumbricoides*
40 (roundworm), hookworm, and *Trichuris trichiura* (whipworm). However, low single-dose cure rates
41 of the benzimidazole drugs, the mainstay of preventative chemotherapy for whipworm, together with
42 parasite drug resistance, mean that current approaches may not be able to eliminate morbidity from
43 Trichuriasis. We are seeking to develop new anthelmintic drugs specifically with activity against
44 whipworm as a priority, and previously identified a hit series of dihydrobenzoxazepinone (DHB)
45 compounds that block motility of *ex vivo* *Trichuris muris*. Here we report a systematic investigation
46 of the structure-activity relationship of the anthelmintic activity of DHB compounds. We synthesised
47 47 analogues, which allowed us to define features of the molecules essential for anthelmintic action,
48 as well as broadening the chemotype by identification of dihydrobenzoquinolinones (DBQ) with
49 anthelmintic activity. We investigated the activity of these compounds against other parasitic
50 nematodes, identifying DHB compounds with activity against *Brugia malayi* and *Heligmosomoides*
51 *polygyrus*. We also demonstrated activity of DHB compounds against the trematode *Schistosoma*
52 *mansoni*, a parasite that causes schistosomiasis. These results demonstrate the potential of DHB and
53 DBQ compounds for further development as broad-spectrum anthelmintics.

54

55 **Author summary**

56 Around a billion people are infected by the soil transmitted helminths *Ascaris*, hookworm and
57 whipworm. In the case of whipworm, the benzimidazole drugs, which are distributed to school
58 children in affected areas, have low cure rates. This means that finding an improved treatment for
59 whipworm is a priority. We previously identified five DHB compounds in a screen for new
60 compounds active against whipworm. Here we systematically dissect these molecules, making 47
61 modified versions of the compounds. This allowed us to define the features of these compounds that
62 are important for activity against whipworm. We also demonstrate activity of DHB compounds
63 against other parasitic nematodes, and against *Schistosoma mansoni*, a trematode parasite. These

64 results show the potential for further development of DHB compounds as broad-spectrum
65 anthelmintics.

66

67 **Introduction**

68 900 million people are infected with soil-transmitted helminths causing a global burden of around two
69 million disability-adjusted life years [1,2]. Because of this, the World Health Organization has set a
70 goal to achieve and maintain elimination of soil-transmitted helminth morbidity by 2030 [3]. Huge
71 mass drug administration efforts are underway, distributing hundreds of millions of doses of
72 benzimidazole drugs (albendazole and mebendazole) to school-age children in affected areas annually
73 or biannually as preventative chemotherapy (PCT).

74 Benzimidazole drugs are partially effective against whipworm (*Trichuris trichiura*) when
75 administered as a course of treatment, reaching cure rates of around 43% [4]. However, for mass drug
76 administration, practicalities and scale mean that only one dose is given. In contrast to *Ascaris*
77 *lumbricoides*, where a single dose of benzimidazole drugs cures around 90-95% of infected
78 individuals, the single dose cure rate for whipworm is low, around 30% [5,6]. The current mass drug
79 administration protocol may therefore not be able to break transmission and reduce the prevalence of
80 moderate to heavy whipworm infections to below 2% as required to eliminate morbidity [3]. Due to
81 the poor single dose efficacy of the benzimidazole drugs against whipworm, there have been
82 extensive efforts to identify more efficacious drug combinations [6]. Of these the most promising to
83 date is a combination of albendazole plus the N-type nicotinic acetylcholine receptor agonist oxantel
84 pamoate, which has a single dose cure rate reported to be between 31 and 83% [7–10]. A second drug,
85 moxidectin, also shows promise to be added to albendazole for improved control of whipworm [7].

86 Of concern, however, is the possibility that drug resistance may become prevalent, derailing the push
87 towards control of whipworm. Currently there is only indirect evidence of this possibility. In a meta-
88 analysis, it has been shown that egg reduction rates and cure rates after albendazole treatment are
89 decreasing over time [11]. Polymorphisms in the beta-tubulin gene that are associated with

90 benzimidazole resistance are found in populations of human whipworm, and the frequency of these
91 polymorphisms increased after albendazole treatment [12,13]. The Starworms project will establish a
92 valuable system to monitor benzimidazole drug efficacy and the potential emergence of anthelmintic
93 resistance due to soil-transmitted helminth control programs [14]. Because of these two problems –
94 low efficacy of existing drugs against whipworm, and concerns about development of resistance to
95 these drugs – we and others have been pursuing a strategy of identifying new anti-whipworm
96 compounds, via a mixture of repurposing and *de novo* small molecule screening [15–22].

97 Beyond soil transmitted helminths (STH), lymphatic filariasis and schistosomiasis are two medically
98 important tissue helminthiases prioritised for global or regional elimination via mass PCT, as outlined
99 in the WHO Roadmap 2030 implementation targets [23]. A related filarial nematode, *Onchocerca*
100 *volvulus*, is also targeted for regional elimination. Reliance on a few, or in the case of onchocerciasis
101 and schistosomiasis, a single chemotherapeutic agent (ivermectin and praziquantel, respectively) used
102 ‘en masse’ for PCT, is a vulnerability of current elimination strategies, considering the potential for
103 development of drug resistance. As with STH, annual or semi-annual mass drug administrations
104 extending upward of 20 years are required to break transmission with current drugs due to incomplete
105 adulticidal / selective larvicidal activity profiles of the implemented anti-filarial or schistosomicidal
106 agents. Alternative strategies, for instance, development of a short-course curative treatment for
107 filariasis, would be a step-change to reduce elimination time frames [24,25].

108 We previously described a hit series of five dihydrobenz[e][1,4]oxazepin-2(3*H*)-one (DHB)
109 compounds with anthelmintic activity against *ex vivo* *T. muris* [18]. Here we report our progress in
110 expanding this hit series and understanding the relationship between structure and anthelmintic
111 activity. We also extend our investigations of the activity of the DHB compounds against *Brugia*
112 *malayi*, a causative agent of lymphatic filariasis, *Heligmosomoides polygyrus bakeri*, a mouse
113 gastrointestinal nematode model, and the human blood fluke, *Schistosoma mansoni*.

114

115 **Materials and Methods**

116 **Ethics statement**

117 All experimental procedures involving *T. muris* were approved by the University of Manchester
118 Animal Welfare and Ethical Review Board and performed within the guidelines of the Animals
119 (Scientific Procedures) Act, 1986.

120 All experiments involving *Brugia malayi* were approved by the ethical committees of the University
121 of Liverpool and Liverpool School of Tropical Medicine (LSTM) and conducted under Home Office
122 Animals (Scientific Procedures) Act 1986 (UK) requirements and the ARRIVE guidelines.

123 The work on *Heligmosomoides polygyrus* was approved by the local veterinary agency, based on
124 Swiss cantonal and national regulations (permission no. 2070).

125 For experiments involving *Schistosoma mansoni*, all procedures performed on mice adhered to the
126 United Kingdom Home Office Animals (Scientific Procedures) Act of 1986 (project licenses PPL
127 40/3700 and P3B8C46FD) as well as the European Union Animals Directive 2010/63/EU and were
128 approved by Aberystwyth University's (AU) Animal Welfare and Ethical Review Body (AWERB).

129

130 **Chemical synthesis**

131 Compounds were synthesised from commercially available starting materials, and fully characterised
132 by Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry. Full experimental details and
133 analytical data are provided in the Supporting Information.

134 **Isolation of *T. muris* adults**

135 Male and female severe combined immunodeficient (SCID) mice were bred in house at the University
136 of Manchester and used at age 8-12 weeks. Mice were maintained at a temperature of 20-22°C in a
137 12h light, 12h dark lighting schedule, in sterile, individually ventilated cages with food and water *ad*
138 *lib.*

139 The parasite was maintained and the infectivity of the administered *T. muris* eggs was assessed as
140 previously described [26,27]. For generation of adult *T. muris* worms 150 infective eggs were given
141 per oral gavage in water to each SCID mouse. 35 days post infection mice were sacrificed via
142 schedule one methods. At necropsy the caecae and colons were removed, opened longitudinally and
143 washed with pre-warmed RPMI-1640 media supplemented with penicillin (500U/ml) and
144 streptomycin (500µg/ml). Adult *T. muris* worms were gently removed using fine forceps under a
145 dissecting microscope and maintained at 37°C in RPMI-1640 media supplemented with penicillin
146 (500U/ml) and streptomycin (500µg/ml).

147 *T. muris* adult motility assay

148 Single adult worms were placed in microplate wells containing 100 µL of RPMI-1640 medium,
149 penicillin (500 U/mL), streptomycin (500 µg/mL) and 1 µl (1% v/v) dimethylsulfoxide (DMSO) or
150 compound dissolved in DMSO. Assay plates were incubated at 37°C with 5% CO₂. The INVAPP
151 system was used to quantify worm motility [28,29]. Movies of the whole plate were recorded (20
152 frames, 100 ms interval) and motility determined by thresholding the variance of each pixel in the
153 image over time [30]. Compounds were initially tested at 100 µM. Those showing activity were also
154 tested at lower concentrations, typically 50 and 75 µM, and EC₅₀ estimates were measured for
155 compounds of interest using the a log-logistic model and the R package *drc* [31].

156 *B. malayi* parasite production

157 The life cycle of *B. malayi* was maintained in *Aedes aegypti* mosquitoes (Liverpool strain) and inbred
158 Mongolian gerbils housed at the Biomedical Services Unit, University of Liverpool under specific
159 pathogen-free conditions. Microfilariae were harvested from experimentally infected Mongolian
160 gerbils via catheterization under anaesthesia and fed to mosquitoes in human blood at 20,000 mf / ml
161 using artificial membranes heated to 37 °C. Mosquitoes were reared for 14 days with daily sugar-
162 water feeding to allow development to larval stage (*BmL3*). At day 14, *BmL3* were collected from
163 infected mosquitoes by stunning at 4 °C, crushing and concentrating using a Baermann's apparatus
164 and RPMI-1640 media. Male IL-4Rα^{-/-}IL-5^{-/-} BALB/c mice (gifted by Prof. Achim Hoerauf,

165 University of Bonn, Germany) aged 6-8 weeks, weighing 18-24 g were infected intraperitoneally with
166 150 *BmL3* and left for 12 weeks to develop to patent adult stage as previously detailed [32].

167 ***B. malayi* microfilaria assay**

168 *Brugia malayi* microfilariae (mf) were harvested from Mongolian gerbils via intraperitoneal lavage
169 and purified using PD-10 columns (Amersham). Mf densities were then adjusted to 8,000 / well in
170 complete medium consisting of RPMI-1640 supplemented with 1% penicillin-streptomycin, 1%
171 amphotericin B and 10% FBS within 96 well plates.

172 33 test compounds (10 mM stock in 100% DMSO) were initially tested against mf. Compounds were
173 diluted to 10 μ M in complete medium and added to the plated mf. Three replicates were used for each
174 compound, and each plate included ivermectin (50 μ M) as a positive control and DMSO (0.5% v/v) as
175 a negative control. Assay plates were incubated for 6 days at 37°C, 5% CO₂. Mf were scored daily for
176 motility as a proxy of nematode health, using a 5 point scoring system (4 = fully motile, 0 = no
177 motility) as described previously [33]. Compounds found to reduce motility were progressed to a
178 secondary screen, whereby the MTT assay was employed at day 6 to assess parasite viability
179 quantitatively. For this, excess media was removed from wells and mf were incubated with 0.5 mg/ml
180 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Merck) in PBS at 37°C for 90
181 min. After washing in PBS and centrifugation, mf pellets were incubated in 100% DMSO for 1 h at
182 37°C to solubilize the blue formazan product. Samples were read at OD 490 nm on a 96-well plate
183 reader (Varioskan, Bio-Rad). Compounds exhibiting the greatest activity on parasite viability were
184 progressed further for drug dose titration assays.

185 ***B. malayi* adult assay**

186 Adult female *B. malayi* of 12-24 weeks of age were isolated from susceptible IL-4R α ^{-/-}IL-5^{-/-}
187 immunodeficient mice, washed in PBS and added to lymphatic endothelial cell co-cultures
188 (HMVECdly; LEC; Lonza) at a density of two parasites per well. Successful test compounds from the
189 mf assay were diluted to 10 μ M and added to the trans-wells in 6 ml endothelial basal media with
190 supplements (EGM-2 MV; Lonza). Twelve replicates (n = 6 wells) were set up per group, with

191 flubendazole (10 μ M; Sigma) and DMSO (0.5% v/v) added as controls. Plates were incubated for 14
192 days at 37 °C, 5% CO₂ with daily motility scoring, as above. Individual parasites were taken for MTT
193 analysis at day 14.

194 *Heligmosomoides polygyrus*

195 *H. polygyrus* larvae (L3) were obtained by filtering the faeces of infected mice and cultivating the
196 eggs on an agar plate for 8-10 days in the dark at 24°C. 30-40 L3 were placed in each well of a 96-
197 well plate for each compound in the presence of 100 μ l RPMI 1640 (Gibco, Waltham MA, USA)
198 culture medium supplemented with 5% amphotericin B (250 μ g/ml, Sigma-Aldrich, Buchs,
199 Switzerland) and 1% penicillin 10,000 U/ml, and streptomycin 10 mg/ml solution (Sigma-Aldrich,
200 Buchs, Switzerland) with the test drugs (100 μ M concentration). Worms were kept at room
201 temperature for 72 h and for evaluation 50-80 μ l of hot water (\approx 80°C) was added to each well and the
202 larvae that responded to this stimulus (the moving worms) were counted. The proportion of larval
203 death was determined. Compounds were tested in duplicate at 100 μ M. Control wells were included
204 in each experiment, which included the highest amount of solvent (1% DMSO).

205 *S. mansoni* Roboworm assay

206 *Biomphalaria glabrata* (NMRI and the previously described pigmented strains [34]) infected
207 previously with *S. mansoni* (Puerto Rican strain) miracidia were exposed for 1.5 hrs under light at 26
208 °C. Cercariae were collected and mechanically transformed into schistosomula as previously
209 described [35]. Mechanically-transformed schistosomula were subsequently prepared for high
210 throughput screening (HTS) on the Roboworm platform according to Crusco *et. al.* [36]. All
211 compounds were tested in duplicate during dose response titrations (50 μ M, 40 μ M, 30 μ M, 20 μ M,
212 10 μ M in 0.625% DMSO). Assay controls included 10 μ M (in 0.625% DMSO) auranofin (positive
213 control; Sigma-Aldrich, UK) and 0.625% DMSO (negative control). Schistosomula phenotype and
214 motility were quantified after 72 hr co-culture with compounds as previously described [37].
215 Compounds passing both phenotype (-0.15) and motility (-0.35) thresholds were classified as hits. Z'
216 scores for all assays were above 0.35 [38].

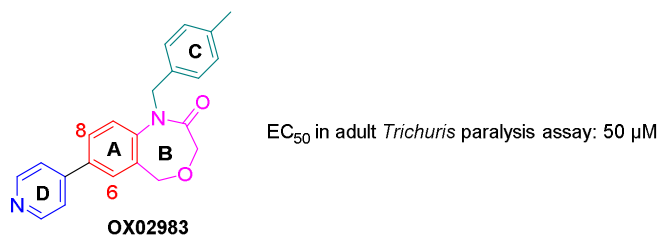
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218 Results

219 Novel DHB Chemistry

220 We have recently reported the identification of five dihydrobenzoxazepinone (DHB) hit compounds
221 as a new family of molecules active against *T. muris* adult motility [18]. Further, one of the
222 compounds **OX02983** was also found to be efficacious at reducing the ability of eggs to establish
223 infection *in vivo*. As we identified a limited number of active DHB family members in the first
224 instance via a library screen, we aimed to investigate the DHB chemotype systematically with the
225 goal of understanding their structure-activity relationships (SARs) and improving potency. **OX02983**
226 was used as a starting point of our investigation.

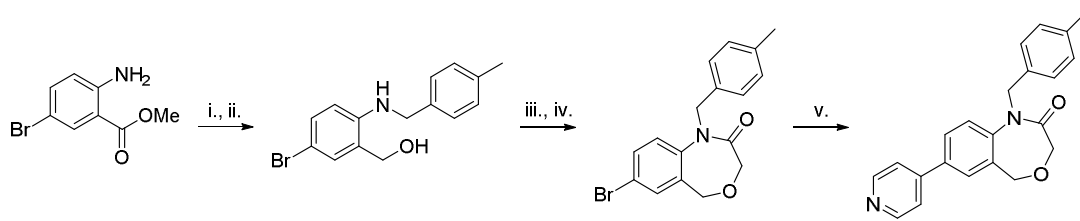
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229 **Fig 1. Structure of OX02983 highlighting the four cycles labelled A-D**

230

231 The synthesis used to prepare **OX02983** was adapted to systematically alter all the different cycles **A-**
232 **D**, as shown in Fig 1. The first step was a reductive amination of the requisite aminobromobenzoate
233 with the desired aldehyde to install cycles **A** and **C**. This was followed by a ring-closure step to
234 generate cycle **B** and finally a cross-coupling reaction to add cycle **D** (Scheme 1).



236 **Scheme 1. Representative scheme for synthesis of DHB compounds.** i. 4-methylbenzaldehyde (1.5
237 eq.), AcOH (0.5 eq.), NaBH(OAc)₃, CH₂Cl₂, 0 °C to rt, 48 h; ii. LiAlH₄ (1 M in THF, 3.5 eq.); iii.
238 chloroacetyl chloride (2.0 eq.), NEt₃ (2.0 eq.), THF, 0 °C to rt, 16 h; iv. NaOH (10 N, aq.), rt, 2 h; v.
239 4-pyridyl-B(OH)₂ (1.1 eq.), Pd(dppf)Cl₂ (5 mol%), K₂CO₃ (3.0 eq.), 1,4-dioxane/H₂O (4:1), 90 °C, 18
240 h.

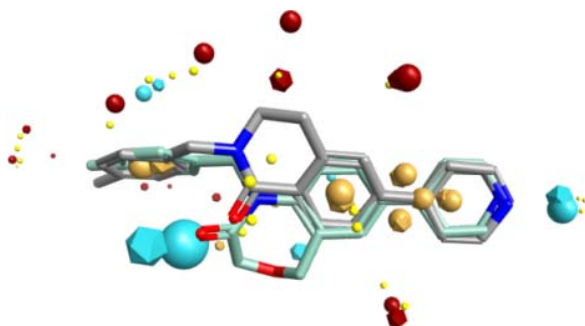
241 It was decided to conduct a systematic structure activity relationship (SAR) investigation and alter the
242 four different cycles within the structure of **OX02983** to understand their importance in the activity
243 against *T. muris* with a view to improving efficacy. As the synthesis is linear, it was logical to
244 investigate from **A** to **D**. We therefore started with core **B**, to ascertain the importance of
245 regiochemistry and relative orientation of the substituents (Table 1). All the prepared compounds
246 were screened using an automated adult *T. muris* motility assay [28] at 100 μM. Active compounds
247 were also tested at lower concentrations and/or an EC₅₀ value determined to assess their relative
248 activity.

249 Using the appropriate starting materials (see S1 File for details of the syntheses), the different
250 structural analogues **OX03701**, **OX03707** (where the 4-pyridyl ring is in position 8 and 6 of the
251 bicyclic core respectively, see Fig 1) and **OX03704** (the reverse amide equivalent of **OX02983**) were
252 prepared using a similar synthesis to **OX02983** (Table 1). Interestingly, none of the structural
253 analogues exhibited any activity in our *ex vivo* adult *T. muris* motility assay, revealing that the
254 regiochemistry within **OX02983** is important for its activity. The next step was to investigate cycle **C**;
255 a small set of amines was used in the reductive amination step to prepare analogues **OX04118**,
256 **OX04120**, **OX02993**, **OX03825**, **OX03144** bearing methyl, cyclopropyl, cyclohexyl, benzyl and *p*-
257 trifluoromethylbenzyl groups respectively. From those, only the cyclohexyl substituted derivative
258 **OX02993** and the *p*-trifluoromethylbenzyl substituted derivative **OX03144** showed activity in the

259 motility assay, with EC₅₀ values of 52 μM and 26 μM respectively. The next step was to vary cycle **D**,
260 while keeping cycles **A-C** constant to allow a comparison with **OX02983**. Suzuki reactions were
261 therefore carried out on the 7-bromo precursor with an array of boronic acids and esters. The
262 regioisomers of the pyridyl ring (**D**) were tolerated with *meta* and *para* giving the best activity.
263 Analogues where the pyridyl ring was replaced with an aryl substituent were all inactive, be they
264 unsubstituted (**OX03596**), substituted with an electron withdrawing group (4-F, **OX03600**), or an
265 electron donating group (4-Me, **OX03601**) (Table 1). Different heterocycles were also trialled in place
266 of the pyridine; a similar level of activity was obtained with the isosteric thiazole (**OX04122**, EC₅₀ of
267 45 μM) and the methylimidazole (**OX04123**, EC₅₀ of 68 μM) analogues. Substituting with a
268 pyrimidine (**OX03705**) led to a loss of activity, leading us to hypothesize that the basicity of the
269 substituent may be of importance to the activity. Following this, we prepared phenylamine and benzyl
270 amine-substituted analogues **OX03824** and **OX03710**, but neither exhibited activity against *T. muris*,
271 suggesting incorporating a linker between cycles **A** and **D** was not tolerated. We then turned our
272 interest to substituted pyridyl, and although the methoxy substituted pyridyl (**OX04116**) was not
273 active, the amino pyridyl **OX04117** displayed modestly improved activity than **OX02983** (EC₅₀ 26
274 μM), which may be related to its moderately higher basicity.

275 In an effort to improve the efficacy further, we looked at more drastic modification to core **B**, by
276 contracting the ring by removing the oxygen atom. Forge (Cresset) was used to overlay **OX02983** and
277 its six-membered ring analogue **OX3699**; a good fit was obtained (~79% similarity) suggesting
278 dihydrobenzoquinolinones (DBQ) as possible candidates for further improvement (Fig 2).

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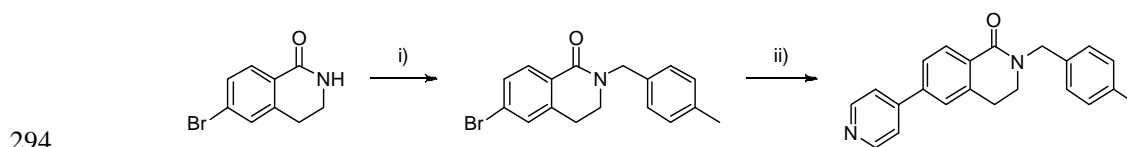
281 **Fig 2.** Overlay of **OX02983** (light blue) and **OX3699** (grey); blue spheres represent negative
282 electrostatic field, red spheres represent positive electrostatic field, brown spheres represent
283 hydrophobicity and small yellow sphere represent the van der Waals force.

284

285 DBQs have been investigated quite extensively in medicinal chemistry; examples have been reported
286 as antiviral agents through inhibition of HIV replication [39,40]. Other analogues were found to
287 inhibit WDR5 protein-protein interactions, leading to inhibition of cancer cell proliferation [41–43].³⁻
288 ⁵

289 It was decided to prepare a small number of compounds only using those substituents and comparable
290 regiochemistry that gave the most potent analogues so far.

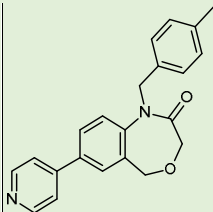
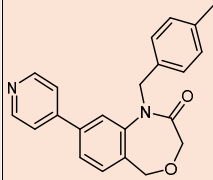
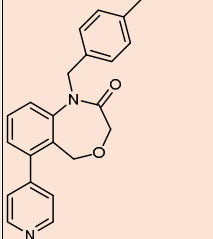
291 The synthesis started with substitution at *N2* of 6-bromo-3,4-dihydroisoquinolinone with 4-
292 methylbenzyl bromide, followed by a Suzuki coupling reaction with the requisite boronic acid to
293 afford the desired ring contracted **OX02983** mimic (Scheme 2).

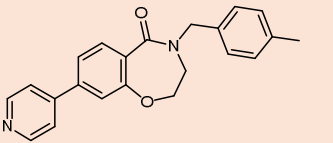
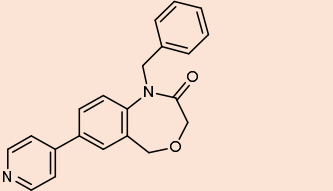
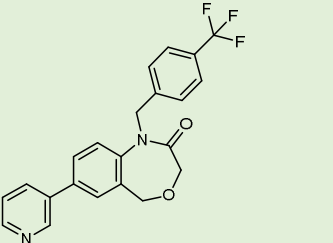
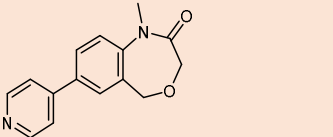
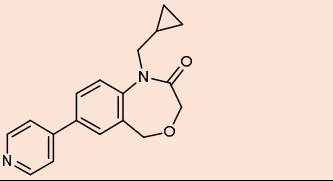
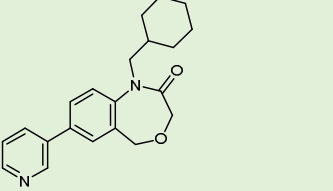
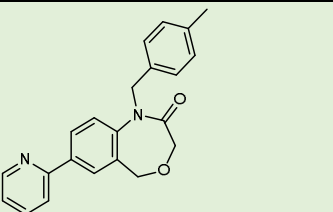
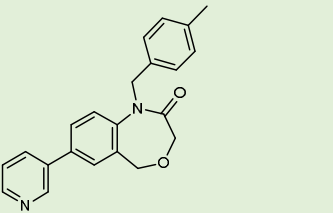


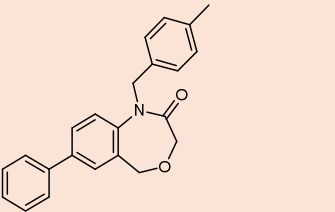
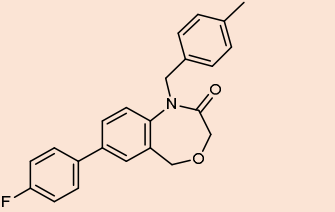
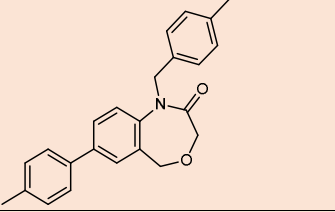
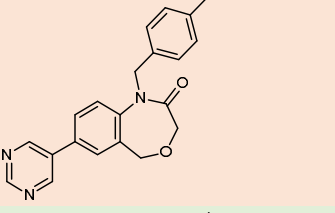
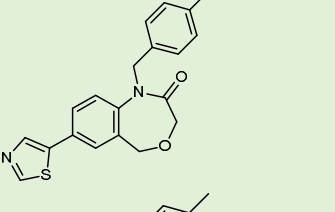
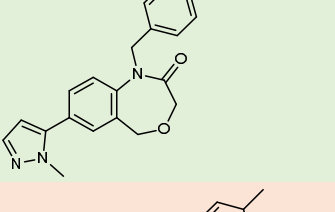
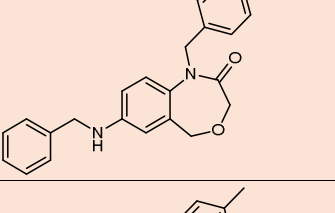
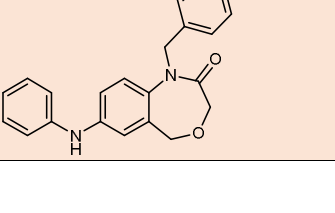
295 **Scheme 2.** Synthesis of **OX3699**: Reagents and conditions: i) 4-methylbenzyl bromide (2.0 eq.), NaH
296 (1.5 eq.), DMF, rt, 16 h (96 %); ii) 4-pyridyl-B(OH)₂ (1.1 eq.), Pd(dppf)Cl₂ (5 mol%), K₂CO₃
297 (3.0 eq.), 1,4-dioxane/H₂O (4:1), 90 °C 18 h.

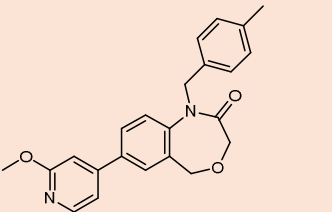
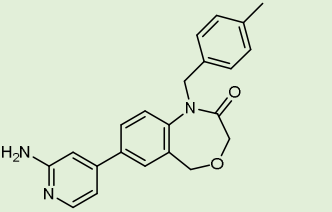
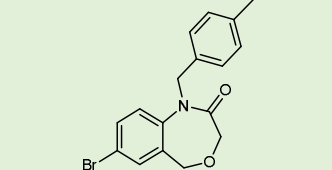
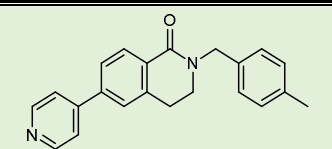
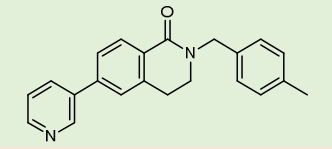
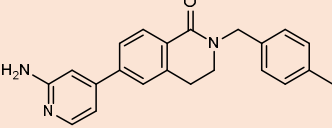
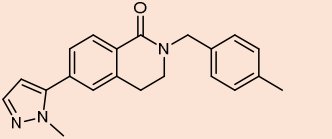
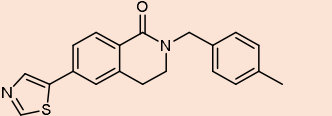
298 The DBQ bearing the 3-and 4-pyridyl substituents (**OX03699** and **OX04236**) were active in the
299 motility assay and led to similar EC₅₀s to the best results from the DHB series (with EC₅₀ values of 21
300 μM and 46 μM respectively). Unfortunately, as soon as we moved away from the simple pyridyl
301 substituent, all activity in the motility assay was lost again. The 2-amino pyrid-5-yl, the best example
302 of ring **D** in the DHB series, was surprisingly inactive (**OX04238** EC₅₀ >100 μM vs. **OX04117** EC₅₀
303 26 μM). Similarly, the methyl imidazole and the thiazole-substituted analogues (**OX04237** and
304 **OX4739** respectively), also exhibited no activity in the motility assay, in contrast to their DHB
305 counterparts suggesting that SARs did not correlate between the DHB and DBQ series. As the best

306 results from the DQB and the DHB series were largely similar, we felt that this alternative core was
307 not going to enhance substantially the potency of the compounds.
308 Collectively, these data have improved our understanding or provided insights into the SARs of the
309 DHB/DQB family of compounds. The structure of cycles **A** and **B** in **OX02983** were found to be
310 critical to activity; variations of the toluyl group for ring **C** generally also led to inactive compounds.
311 Some variations of cycle **D** were tolerated, and there appeared to be a preference for a basic site
312 within the substituent. However, although we were able to alter the structure resulting in loss of
313 activity, we were unable to improve, only retain, activity.
314 Apart from the representative compounds presented in Table 1, further similar analogues and all
315 synthetic precursors were prepared and tested (S2 File). Together it gave us a library of 47 compounds
316 that could then be used against different parasite species to understand whether these compound series
317 showed broad-spectrum anthelmintic activity.
318
319

Compound	Structure	EC ₅₀ (μM) in <i>T. muris</i> adult motility assay
OX02983		50
OX03701		no activity
OX03707		≥ 100

OX03704		≥ 75
OX03825		≥ 75
OX03144		26
OX04118		≥ 75
OX04120		≥ 75
OX02993		52
OX04115		72
OX03153		57

OX03596		no activity
OX03600		no activity
OX03601		no activity
OX03705		no activity
OX04122		45
OX04123		68
OX03710		no activity
OX03824		no activity

OX04116		no activity
OX04117		26
OX03146		35
OX03699		21
OX04236		42
OX04238		no activity
OX04237		no activity
OX04239		no activity

320

321 **Table 1. Structures and EC₅₀ of representative DHB compounds in the *T. muris* adult motility**
 322 **assay.** All compounds investigated in this study are described in the S2 File. No activity means no
 323 clear reduction in motility when tested at 100 μM. Where an EC₅₀ estimate is shown, it was calculated
 324 using a log-logistic model using the R package drc [31].

325

326 DHB compounds are active in models of a range of helminth infections

327 Whipworm is only one of many widely prevalent human helminth infections, and there are continuing
328 efforts to improve drug treatments for these diseases. There have been recent successes, such as the
329 approval of the veterinary medicine moxidectin for onchocerciasis [44], and the establishment of the
330 triple therapy albendazole, diethylcarbamazine citrate plus ivermectin as an improved microfilaricide
331 treatment for lymphatic filariasis suitable for mass drug administration [45,46]. However, sub-optimal
332 efficacy, problematic contraindications, and concerns that mass drug administration could lead to the
333 spread of drug resistance, mean that repurposing of veterinary anthelmintics, improving drug
334 combinations, and the development of new anthelmintics remain priorities [47–49].

335 Development of a new anthelmintic is a long and expensive process, and funding for neglected
336 tropical diseases is limited. Furthermore, multiple parasitic helminths, for example the soil transmitted
337 nematodes, *Ascaris*, *Trichuris* and the human hookworms, the vector transmitted filarial nematodes,
338 and the *Schistosoma* trematodes, are often endemic in the same regions. It would, therefore, be helpful
339 if a new drug would have activity against several target species and which worked across the
340 Nematoda and Platyhelminthes phyla. We therefore wanted to investigate whether the DHB series of
341 compounds had a range of activities beyond *Trichuris*.

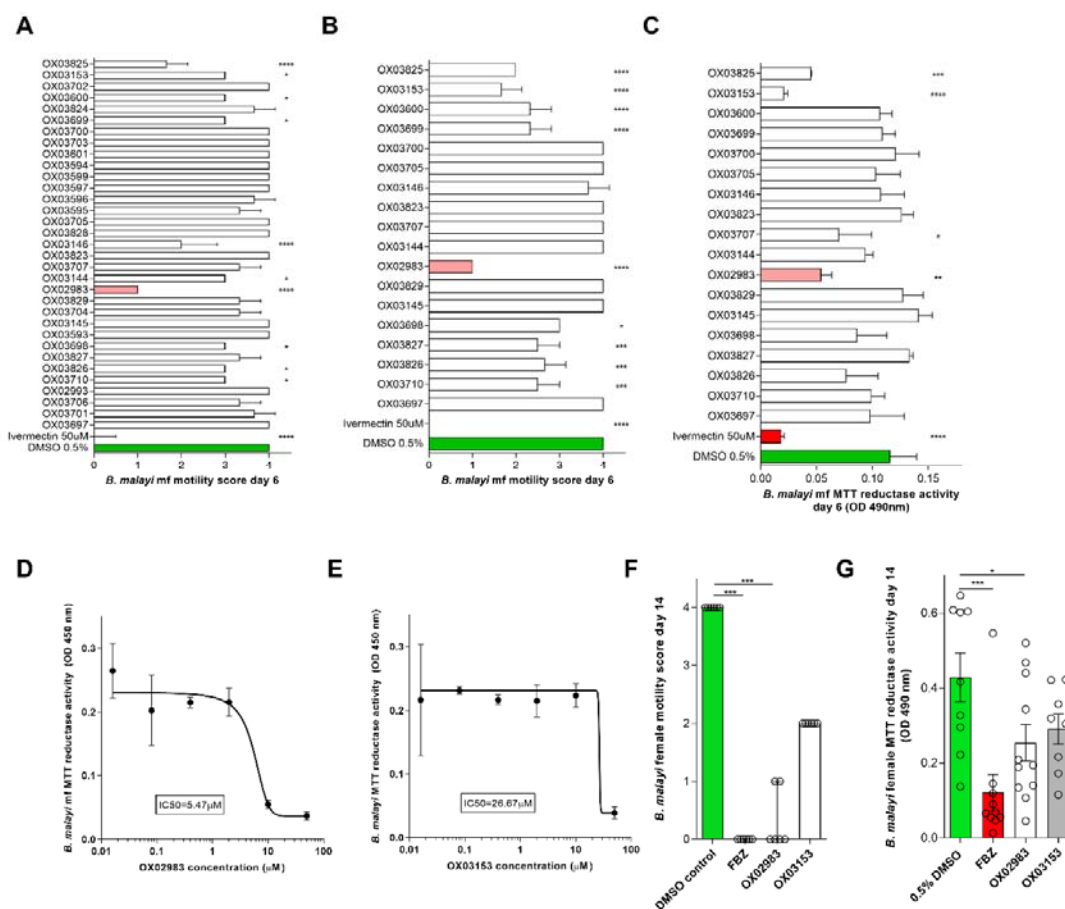
342 Activity against *B. malayi*

343 *B. malayi* is one of the tissue dwelling nematode parasites responsible for human lymphatic filariasis
344 [50]. We first examined single dose efficacy of 33 DHB compounds at 10 μ M against the *B. malayi*
345 mf larval stage, with motility scored every 24 hours. The results after six days are shown in Fig 3A.
346 Ivermectin was used as a positive control in the mf assay. **OX02983** showed the most promise in this
347 assay, reducing average motility to a score of 1. From this primary screen, 15 compounds that were
348 determined to significantly impact *B. malayi* mf motility, plus an additional 5 with no discernible
349 effect, were retested in a secondary screen (Fig 3B). These results confirmed the significant reduction
350 in motility caused by 11 compounds and confirmed the paralytic effect of **OX02983**. After six days
351 drug exposure, mf were also tested for metabolic activity, a measure of parasite viability, using the
352 MTT assay (Fig 3C). **OX02983** and **OX03153**, in particular, showed activity in this assay,

353 significantly reducing *B. malayi* mf MTT reductase activity on average by 53% and 82%, respectively
354 (1-way ANOVA with Holm-Sidak's multiple comparison tests, $P < 0.01$ and $P < 0.0001$). To determine
355 the dose-dependent efficacy of **OX02983** and **OX03153**, they were tested in a concentration response
356 6-day experiment (dose range 0.016-50 μM) using MTT reductase activity as a quantitative viability
357 readout (Fig 3D,E). From this an EC_{50} concentration of 5.5 μM was determined for **OX02983** and
358 26.7 μM for **OX03153**.

359 Due to their efficacy against *B. malayi* mf, **OX02983** and **OX03153** were advanced for *in vitro*
360 activity against adult *B. malayi*, utilising a novel, long-term adult worm lymphatic endothelial cell
361 bilayer co-culture system. Adult female *B. malayi* exposed to vehicle control retained full survival
362 and motility in culture over 14 days whereas the positive control, flubendazole (10 μM) mediated
363 complete paralytic activity by day 14 (Kruskal Wallis with Dunn's multiple comparisons tests,
364 $P < 0.001$) and significantly reduced metabolic activity by an average of 72% (1-way ANOVA with
365 Holm-Sidak's multiple comparison tests, $P < 0.001$) (Fig 3F-G). **OX02983** (10 μM) also mediated
366 significant anti-filarial activities against adult *B. malayi* by day 14. Motility was completely hindered
367 in 4/6 adult parasites by **OX02983** (Kruskal Wallis with Dunn's multiple comparisons tests, $P < 0.001$),
368 whilst **OX03153** mediated a 50% partial reduction in adult motility. **OX02983** also significantly
369 impacted on adult female *B. malayi* metabolic activity, on average by 41% (1-way ANOVA with
370 Holm-Sidak's multiple comparison tests, $P < 0.05$). Taken together, these results are encouraging
371 because they show that compounds that are active against *T. muris* (a clade I nematode according to
372 the phylogeny of Blaxter) are also active against evolutionarily-distant nematodes, as *B. malayi* is a
373 clade III nematode [51].

374



375

376 **Fig 3. Activity of 33 DHB compounds against *B. malayi* microfilariae and adults.** (A) Primary
 377 screen – assessment of *B. malayi* mf motility (5 point scoring system) after six days continuous
 378 exposure to 35 test compound screened at 10 μ M in triplicate. Ivermectin (50 μ M) was the positive
 379 control. (B) Confirmatory mf motility and (C) metabolic activity screening of 15 active compounds
 380 identified in (A) and five inactive compounds (10 μ M in triplicate). (D, E) 50% inhibitory
 381 concentration (EC₅₀) assays of active compounds **OX02983** and **OX03153** on *B. malayi* microfilarial
 382 metabolic activity after 6-day continuous exposure. Metabolic activity (C-E) was assessed by
 383 colorimetric MTT assay, data is optical density of mf extracts measured at 490nm. (F) Effects on adult
 384 female *B. malayi* motility and (G) metabolic activity following 14 day continuous exposure to
 385 **OX02983** or **OX03153** (10 μ M). Flubendazole (10 μ M) was used as a positive control in the assay.
 386 Data plotted is mean \pm SD of 3 replicates (A-E) median and range of 6 replicates (F) and mean \pm
 387 SEM of 9-11 replicates (G). Significant differences were determined by 1-way ANOVA with Holm-

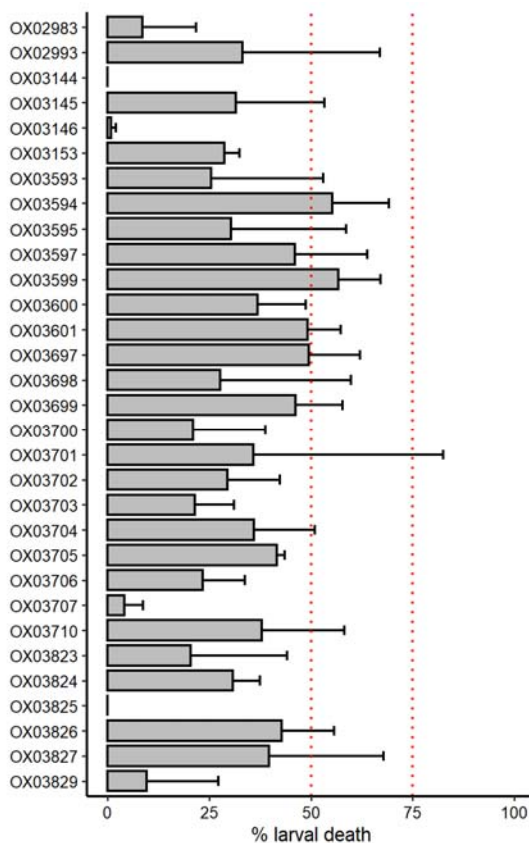
388 Sidak multiple comparisons test (A-C and G) or Kruskal-Wallis with Dunn's multiple comparisons
389 test (F). Significance is indicated ****P<0.0001, ***P<0.001, **P<0.01 and *P<0.05.

390

391 Activity against *H. polygyrus*

392 *H. polygyrus bakeri* is an intestinal nematode parasite of laboratory mice [52]. It is a strongylid
393 nematode, related to human hookworm species. 31 DHB compounds were tested at 100 μ M against *ex*
394 *vivo* *H. polygyrus* L3 stage worms (n = 2). The results are shown in Fig 4. The cut-off used to
395 determine hits in this assay is 50% larval death [17]. Two compounds, **OX03594** and **OX03599**,
396 exceeded this level of larval death and were therefore considered active. They did not however reach
397 the threshold for good activity (75%). Given the modest activity of these compounds against *H.*
398 *polygyrus* we have not further pursued this direction at this point. Activity of DHB compounds
399 against nematodes in three of the five clades of the phylum Nematoda, according to the phylogeny of
400 Blaxter, supports the potential for development of a pan-nematode control agent from this compound
401 series [51].

402



403

404 **Fig 4. Measurement of the activity of 31 DHB compounds against *H. polygyrus* L3 stage worms.**

405 Larval death is measured as the proportion of worms that respond to stimulus. Compounds were
406 tested in duplicate at 100 μ M. Dashed lines indicates the cut-off (50%) used to determine hits in this
407 assay and the cut-off for good activity (75%) [17]: <50%, not active, 50-75% moderate activity,
408 >75% good and >90% excellent activity.

409

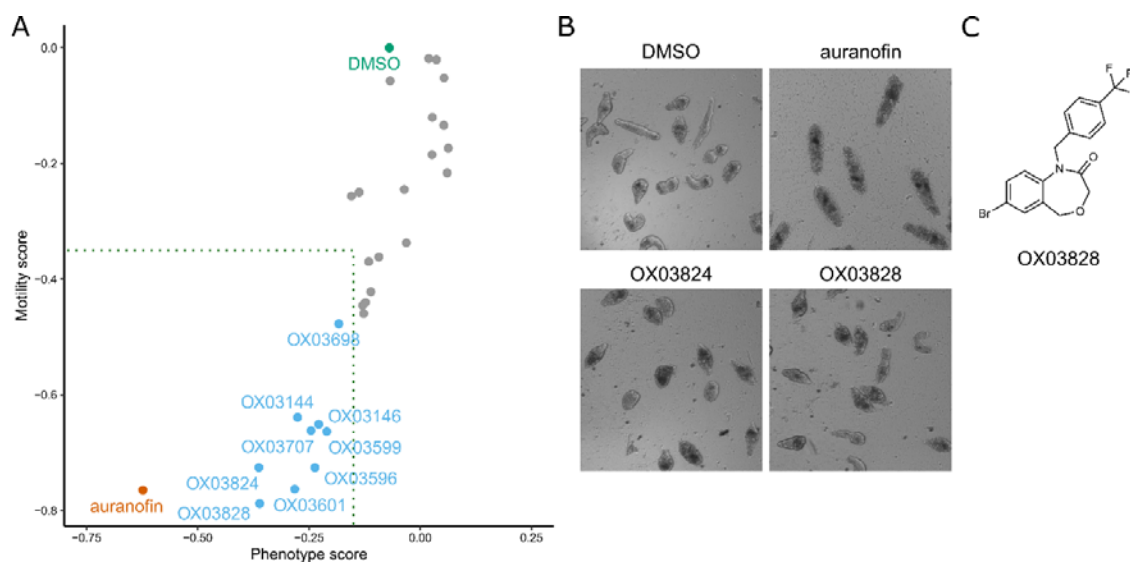
410 Activity against *S. mansoni* schistosomula

411 Compared to *T. muris*, *H. polygyrus* and *B. malayi*, which are all parasitic worms within the phylum
412 Nematoda, *S. mansoni* is a more evolutionary distinct helminth – a trematode within the
413 Platyhelminthes phylum. It is a human parasite that infects around 150 million people, causing
414 schistosomiasis [1]. Praziquantel, an N-acylated quinoline-piperazinone, is the basis of
415 schistosomiasis treatment and is safe and efficacious against adult worms of all *Schistosoma* spp. as a
416 monotherapy. However, there is concern about the emergence of drug resistance [53] and praziquantel

417 has lower efficacy against juvenile forms, so immature parasites may survive drug exposure and
418 continue the infection.

419 We screened 30 DHB compounds against *S. mansoni* schistosomula at 50 μ M using the RoboWorm
420 system. This is an imaging-based screen that measures two parameters, motility and “phenotype,” an
421 assessment of morphological and other features [37]. Auranofin, an inhibitor of *S. mansoni*
422 thioredoxin glutathione reductase (TGR) activity [54] was the positive control in this experiment. The
423 results are shown in Fig 5. The cut-offs for defining hit compounds in this assay have been previously
424 defined [37,55]. Nine compounds were hits in this assay for both motility and phenotype
425 measurements. Concentration-response curves were measured for these compounds (Table 2), with
426 EC₅₀ values in the range 14-41 μ M. It is encouraging that DHB compound series members show
427 activity against such evolutionarily distant pathogens to whipworm, particularly as DHB compounds
428 show little or no cytotoxicity in mammalian cell culture – so these compounds are not broadly toxic
429 [18].

430



431

432 **Fig 5. Measurement of the activity of 30 DHB compounds against *S. mansoni* schistosomula**
433 **using the RoboWorm platform.** (A) Each point is the measured effect of one compound on the two
434 parameters – motility and phenotype. The phenotype score is calculated by a computational model
435 that assesses morphological and texture properties of the schistosomula [37]. Compounds were

436 screened at 50 μ M. Auranofin was the positive control (screened at 10 μ M). Dotted box indicates the
437 threshold for activity in this assay: -0.15 for phenotype and -0.35 for motility; compounds must be
438 below this threshold for both parameters to be considered a hit [37,55]. Compounds were screened in
439 duplicate on two or three separate occasions and the data represents the average scores of these
440 experiments. (B) Representative images of schistosomula treated with controls, **OX03824** and
441 **OX03828**. (C) Structure of **OX03828**. The structure of **OX03824** is shown in Table 1.

442

443

Compound	EC ₅₀ in <i>S. mansoni</i> schistosomula Roboworm assay (μ M)	
	phenotype score	motility score
OX03144	28.4	28.0
OX03146	25.5	25.6
OX03596	29.1	26.8
OX03599	14.2	18.3
OX03601	24.4	20.6
OX03698	28.5	26.6
OX03707	32.7	29.3
OX03824	40.9	36.8
OX03828	25.7	20.7

444

445 **Table 2. EC₅₀ values for compounds active in the *S. mansoni* schistosomula Roboworm assay.**

446 Compounds were screened at 10, 20, 30, 40 and 50 µM and EC₅₀ values were calculated for each of
447 the screening parameters, phenotype and motility.

448

449

450 **Discussion**

451 **Investigation of the DHB structure-activity relationship**

452 We previously identified a small hit series of five DHB compounds with activity against *T. muris*
453 adult motility [18]. In medicinal chemistry, it is important to understand how variations in the
454 structure of the compound affect activity, as this allows us to discover the critical aspects of the
455 compound for target binding, with the overall aim of increasing potency as well as improving
456 physicochemical properties. We therefore embarked upon a systematic, structure-activity relationship
457 investigation, taking advantage of the convenient synthesis of the DHBs, which allowed us to
458 systematically alter the four cyclic components of this class of compounds. A total of 47 variant
459 compounds were synthesised in this work.

460 This work has enabled us to define certain essential features of the anti-whipworm DHB compounds.
461 The 4-pyridyl ring (cycle D in Fig 1) must be in the 7 position, unlike the analogues **OX03701** and
462 **OX03707**. The amide moiety of the oxazepinone ring must be as in **OX02983**, and not as in
463 **OX03704**. The oxazepinone nitrogen can be substituted with methylbenzyl, cyclohexyl and *p*-
464 trifluoromethylbenzyl (**OX02983**, **OX02993**, and **OX03144**), but not methyl, cyclopropyl, or benzyl
465 groups. We also investigated in detail the replacement of cycle B. We found that removal of the
466 oxygen from the DHB core was also consistent with similar activity to **OX02983** – the
467 dihydrobenzoquinolinone compounds **OX03699** and **OX04236** had EC₅₀ values of 21 and 42 µM
468 respectively.

469 **Targeting multiple helminth species with DHB family members**

470 Despite being unable to improve efficacy against *Trichuris* substantially through structural
471 modifications, we were able to demonstrate activity of our compounds against other helminth
472 parasites. In drug discovery for NTDs, pan-anthelmintic activity is desirable given that polyparasitism
473 in the target population is the norm. Thus, being able to target multiple species of helminths with a
474 single drug administered via mass drug administration programmes is of significant benefit. Of
475 particular note was the commonality in DHB compounds active against *T. muris* that were also active
476 against the tissue dwelling nematode parasite *B. malayi*. The ability of the DHB compounds to act
477 against different clades within the nematode phylum is not unprecedented, indeed the co-
478 administration of albendazole with ivermectin is currently advocated for control of *Trichuris*, and the
479 same drug combination (in some situations supplemented with diethylcarbamazine) is widely used
480 against lymphatic filariasis [46]. Indeed, the large-scale efforts to treat lymphatic filariasis have
481 indirectly enhanced the number of people being treated for soil transmitted helminths [56]. Similarly,
482 the alternative drug combination of albendazole and moxidectin is also being explored for the
483 treatment of Trichuriasis given that moxidectin is an approved treatment for onchocerciasis [57].

484 In contrast, there are currently no drugs used in MDA that have demonstrated cross-phyla efficacy
485 against both schistosomes and nematodes. Currently only praziquantel is used for preventative
486 chemotherapy against schistosomes, although co-administration with albendazole is recommended
487 where STHs are co-endemic [58]. Therefore it was notable that DHB family members could work
488 across phyla, showing some activity against both schistosomes and nematodes.

489 Conclusions

490 In this study we have investigated the structure-activity relationship of the DHB compounds, defined
491 essential features for anthelmintic action, and broadened the active series by the discovery of
492 dihydrobenzoquinolinone compounds with activity against *T. muris* adult motility. We have also
493 demonstrated that DHB and related compounds have activity against multiple helminths across
494 different phyla – against the nematodes *B. malayi* and *H. polygyrus* as well as *T. muris*, and against
495 the trematode *S. mansoni*. What we have not achieved however, is the substantive improvement in
496 potency from the 20-50 μ M range that would be desirable to progress this series with confidence to *in*

497 *vivo* testing. Open science, where information is disclosed more freely than in traditional models, is
498 proposed to accelerate drug discovery and make it more cost efficient, especially in the context of
499 neglected diseases [59,60]. We have therefore decided to report our progress at this point. We note
500 that we do not yet know the target of the DHB/DBQ compounds in helminths. Identifying this target
501 may facilitate the boost in activity we are striving for.

502

503 **Acknowledgments**

504 JDT, MJT and AEM were supported by a National Centre for Replacement, Refinement and
505 Reduction of Animals in Research Studentship (NC3R Studentship NC/M00175X/1) and a Bill and
506 Melinda Gates Foundation award to the Liverpool School of Tropical Medicine (BMGF
507 OPP1054324).

508 FAP and DBS are supported by Medical Research Council grant MR/N024842/1 and a
509 UCL/Wellcome Trust Translational Partnership Pilot Grant.

510 JFT, HW and KFH acknowledge the Welsh Government Life Sciences Research Network Wales and
511 the Wellcome Trust Pathfinder (201008/Z/16/Z) schemes for financial support of the Roboworm
512 platform.

513

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717 **Supporting information Captions**

718 **S1 File. Supporting information for synthetic chemistry**

719 **S2 File. Summary table of compound structures and assay results.** Shaded results are those
720 compounds that were active in each assay.

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