Structural requirements for dihydrobenzoxazepinone anthelmintics: actions against medically important and model parasites - *Trichuris muris*, *Brugia malayi* and *Heligmosomoides polygyrus*

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

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Abstract

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

Introduction

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

Benzimidazole drugs are partially effective against whipworm *(Trichuris trichiura)* when administered as a course of treatment, reaching cure rates of around 43% [4]. However, for mass drug administration, practicalities and scale mean that only one dose is given. In contrast to the *Ascaris*

lumbricoides, where a single dose of benzimidazole drugs cures around 90-95% of infected individuals, the single dose cure rate for whipworm is low, around 30% [5,6]. The current mass drug administration protocol may therefore not be able to break transmission and reduce the prevalence of moderate to heavy whipworm infections to below 2% as required to eliminate morbidity [3]. Due to the poor single dose efficacy of the benzimidazole drugs against whipworm, there have been extensive efforts to identify more efficacious drug combinations [6]. Of these the most promising to date is a combination of albendazole plus the N-type nicotinic acetylcholine receptor agonist oxantel pamoate, which has a single dose cure rate reported to be between 31 and 83% [7–10]. A second veterinary drug, moxidectin, also shows promise to be added to albendazole for improved control of whipworm [7].

Of concern, however, is the possibility that drug resistance may become prevalent, derailing the push towards control of whipworm. Currently there is only indirect evidence of this possibility. In a meta-analysis, it has been shown that egg reduction rates and cure rates after albendazole treatment are decreasing over time [11]. Polymorphisms in the beta-tubulin gene that are associated with benzimidazole resistance are found in populations of human whipworm, and the frequency of these polymorphisms increased after albendazole treatment [12,13]. The Starworms project will establish a valuable system to monitor benzimidazole drug efficacy and the potential emergence of anthelmintic resistance due to soil-transmitted helminth control programs [14]. Because of these two problems – low efficacy of existing drugs against whipworm, and concerns about development of resistance to these drugs – we and others have been pursuing a strategy of identifying new anti-whipworm compounds, via a mixture of repurposing and *de novo* small molecule screening [15–22].

Beyond soil transmitted helminths (STH), lymphatic filariasis and schistosomiasis are two medically important tissue helminthiases prioritised for global or regional elimination via mass PCT, as outlined in the WHO Roadmap 2030 implementation targets [23]. A related filarial nematodiasis, onchocerciasis, is also targeted for regional elimination. Reliance on a few, or in the case of onchocerciasis and schistosomiasis, a single chemotherapeutic agent (ivermectin and praziquantel, respectively) used ‘en masse’ for PCT, is a vulnerability of current elimination strategies, considering

the potential for development of drug resistance. As with STH, annual or semi-annual mass drug administrations extending upward of 20 years are required to break transmission with current drugs due to incomplete adulticidal / selective larvicidal activity profiles of the implemented anti-filarial or schistosomicidal agents. Alternative strategies, for instance, development of a short-course curative treatment for filariasis, would be a step-change to reduce elimination time frames.

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

Materials and Methods

Ethics statement

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

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

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

Chemical synthesis

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

Isolation of *T. muris* adults

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

The parasite was maintained and the infectivity of the administered *T. muris* eggs was assessed as previously described [24,25]. For generation of adult *T. muris* worms 150 infective eggs were given per oral gavage in water to each SCID mouse. 35 days post infection mice were sacrificed via schedule one methods. At necropsy the caecae and colons were removed, opened longitudinally and washed with pre-warmed RPMI-1640 media supplemented with penicillin (500U/ml) and streptomycin (500µg/ml). Adult *T. muris* worms were gently removed using fine forceps under a dissecting microscope and maintained at 37°C in RPMI-1640 media supplemented with penicillin (500U/ml) and streptomycin (500µg/ml).

T. muris adult motility assay

Single adult worms were placed in microplate wells containing 100 µL of RPMI-1640 medium, penicillin (500 U/mL), streptomycin (500 µg/mL) and 1 µl (1% v/v) dimethylsulfoxide (DMSO) or compound dissolved in DMSO. Assay plates were incubated at 37°C with 5% CO_2. The INVAPP system was used to quantify worm motility [26]. Movies of the whole plate were recorded (20 frames, 100 ms interval) and motility determined by thresholding the variance of each pixel in the image over time [27]. Compounds were initially tested at 100 µM. Those showing activity were also tested at lower concentrations, typically 50 and 75 µM, and EC_{50} estimates were measured for compounds of interest using the a log-logistic model and the R package *drc* [28].

B. malayi parasite production

The life cycle of *B. malayi* was maintained in *Aedes aegypti* mosquitoes (Liverpool strain) and inbred Mongolian gerbils housed at the Biomedical Services Unit, University of Liverpool under specific pathogen-free conditions. Microfilariae were harvested from experimentally infected Mongolian gerbils via catheterization under anaesthesia and fed to mosquitoes in human blood at 20,000 mf / ml using artificial membranes heated to 37 °C. Mosquitoes were reared for 14 days with daily sugar-water feeding to allow development to larval stage (*Bm*L3). At day 14, *Bm*L3 were collected from infected mosquitoes by stunning at 4 °C, crushing and concentrating using a Baermann's apparatus and RPMI-1640 media. Male IL-4Rα$^{-/-}$IL-5$^{-/-}$ BALB/c mice (gifted by Prof. Achim Hoerauf, University of Bonn, Germany) aged 6-8 weeks, weighing 18-24 g were infected intraperitoneally with 150 *Bm*L3 and left for 12 weeks to develop to patent adult stage as previously detailed [29].

B. malayi microfilaria assay

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

33 test compounds (10 mM stock in 100% DMSO) were initially tested against mf. Compounds were diluted to 10 µM in complete medium and added to the plated mf. Three replicates were used for each compound, and each plate included ivermectin (50 µM) as a positive control and DMSO (0.5% v/v) as a negative control. Assay plates were incubated for 6 days at 37°C, 5% CO_2. Mf were scored daily for motility as a proxy of nematode health, using a 5 point scoring system (4 = fully motile, 0 = no motility) as described previously [30]. Compounds found to reduce motility were progressed to a secondary screen, whereby the MTT assay was employed at day 6 to assess parasite viability quantitatively. For this, excess media was removed from wells and mf were incubated with 0.5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Merck) in PBS at 37°C for 90

164 min. After washing in PBS and centrifugation, mf pellets were incubated in 100% DMSO for 1 h at
165 37°C to solubilize the blue formazan product. Samples were read at OD 490 nm on a 96-well plate
166 reader (Varioskan, Bio-Rad). Compounds exhibiting the greatest activity on parasite viability were
167 progressed further for drug dose titration assays.

168 *B. malayi* adult assay

169 Adult female *B. malayi* of 12-24 weeks of age were isolated from susceptible IL-4Rα$^{-/-}$IL-5$^{-/-}$
170 immunodeficient mice, washed in PBS and added to lymphatic endothelial cell co-cultures
171 (HMVECdly; LEC; Lonza) at a density of two parasites per well. Successful test compounds from the
172 mf assay were diluted to 10 µM and added to the trans-wells in 6 ml endothelial basal media with
173 supplements (EGM-2 MV; Lonza). Twelve replicates (n = 6 wells) were set up per group, with
174 flubendazole (10 µM; Sigma) and DMSO (0.5% v/v) added as controls. Plates were incubated for 14
175 days at 37 °C, 5% CO_2 with daily motility scoring, as above. Individual parasites were taken for MTT
176 analysis at day 14.

177 *Heligmosomoides polygyrus*

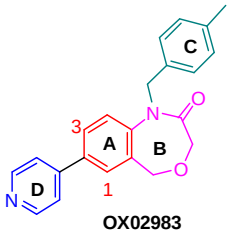
178 *H. polygyrus* larvae (L3) were obtained by filtering the faeces of infected mice and cultivating the
179 eggs on an agar plate for 8-10 days in the dark at 24°C. 30-40 L3 were placed in each well of a 96-
180 well plate for each compound in the presence of 100 µl RPMI 1640 (Gibco, Waltham MA, USA)
181 culture medium supplemented with 5% amphotericin B (250 µg/ml, Sigma-Aldrich, Buchs,
182 Switzerland) and 1% penicillin 10,000 U/ml, and streptomycin 10 mg/ml solution (Sigma-Aldrich,
183 Buchs, Switzerland) with the test drugs (100 µM concentration). Worms were kept at room
184 temperature for 72 h and for evaluation 50-80 µl of hot water (≈80°C) was added to each well and the
185 larvae that responded to this stimulus (the moving worms) were counted. The proportion of larval
186 death was determined. Compounds were tested in duplicate at 100 µM. Control wells were included
187 in each experiment, which included the highest amount of solvent (1% DMSO).

188

Results

Novel DHB Chemistry

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

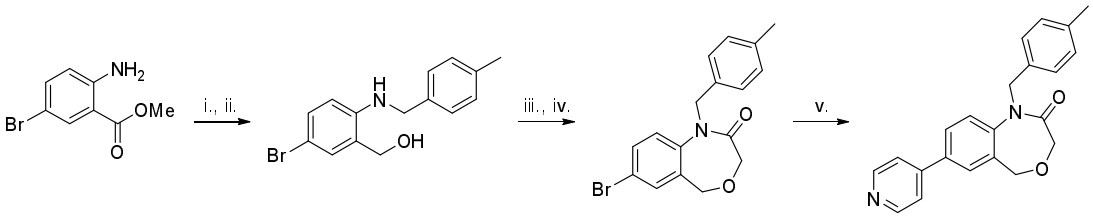


OX02983

EC_{50} in adult Tricuris paralysis assay: 50 uM

Figure 1. Structure of OX02983 highlighting the four cycles labelled A-D

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



Scheme 1. Representative scheme for synthesis of DHB compounds. i. 4-methylbenzaldehyde (1.5 eq.), AcOH (0.5 eq.), $NaBH(OAc)_3$, CH_2Cl_2, 0 °C to RT, 48 h; ii. $LiAlH_4$ (1 M in THF, 3.5 eq.); iii.

chloroacetyl chloride (2.0 eq.), NEt_3 (2.0 eq.), THF, 0 °C to rt, 16 h; iv. NaOH (10 N, aq), rt, 2 h; v. 4-pyridyl-$B(OH)_2$ (1.1 eq.), $Pd(dppf)Cl_2$ (5 mol%), K_2CO_3 (3 eq.), 1,4-dioxane/H_2O (,4:1), 90 ^oC 18 h.

It was decided to conduct a systematic structure activity relationship (SAR) investigation and alter the four different cycles within the structure of **OX02983** to understand their importance in the activity against *T. muris* with a view to improving efficacy. As the synthesis is linear, it was logical to investigate from **A** to **D**. We therefore started with core **B**, to ascertain the importance of regiochemistry and relative orientation of the substituents (Table 1). All the prepared compounds were screened using an automated adult *T. muris* motility assay [26] at 100 µM. Active compounds were also tested at lower concentrations and/or an EC_{50} value determined to assess their relative activity.

Using the appropriate starting materials (see S1 File for details of the syntheses), the different structural analogues **OX03701**, **OX03707** (where the 4-pyridyl ring is in position 8 and 6 of the bicyclic core respectively, see Figure 1) and **OX03704** (the reverse amide equivalent of **OX02983**) were prepared using a similar synthesis to **OX02983** (Table 1). Interestingly, none of the structural analogues exhibited any activity in our *ex vivo* adult *T. muris* motility assay, revealing that the regiochemistry within **OX02983** is important for its activity. The next step was to investigate cycle **C**; a small set of amines was used in the reductive amination step to prepare analogues **OX04118**, **OX04120**, **OX02993**, **OX03825**, **OX03144** bearing methyl, cyclopropyl, cyclohexyl, benzyl and *p*-trifluoromethylbenzyl groups respectively. From those, only the cyclohexyl substituted derivative **OX02993** and the *p*-trifluoromethylbenzyl substituted derivative **OX03144** showed activity in the motility assay, with EC_{50} values of 52 µM and 26 µM respectively. The next step was to vary cycle **D**, while keeping cycles **A**-**C** constant to allow a comparison with **OX02983**. Suzuki reactions were therefore carried out on the 7-bromo precursor with an array of boronic acids and esters. The regioisomers of the pyridyl ring (**D**) were tolerated with meta and para giving the best activity. Analogues where the pyridyl ring was replaced with an aryl substituent were all inactive, be they unsubstituted (**OX03596**), substituted with an electron withdrawing group (4-F, **OX03600**), or an electron donating group (4-Me, **OX03601**) (Table 1). Different heterocycles were also trialled in place

of the pyridine; a similar level of activity was obtained with the isosteric thiazole (**OX04122**, EC_{50} of 45 µM) and the methylimidazole (**OX04123**, EC_{50} of 68 µM) analogues. Substituting with a pyrimidine (**OX03705**) led to a loss of activity, leading us to hypothesize that the basicity of the substituent may be of importance to the activity. Following this, we prepared phenylamine and benzyl amine-substituted analogues **OX03824** and **OX03710**, but neither exhibited activity against *T. muris*, suggesting incorporating a linker between cycles **A** and **D** was not tolerated. We then turned our interest to substituted pyridyl, and although the methoxy substituted pyridyl (**OX04116**) was not active, the amino pyridyl **OX04117** displayed modestly improved activity than **OX02983** (EC_{50} 26 µM), which may be related to its moderately higher basicity.

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

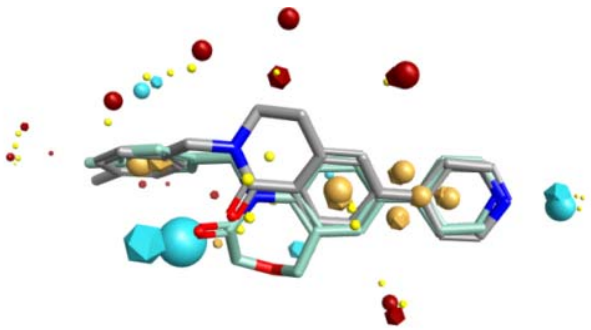


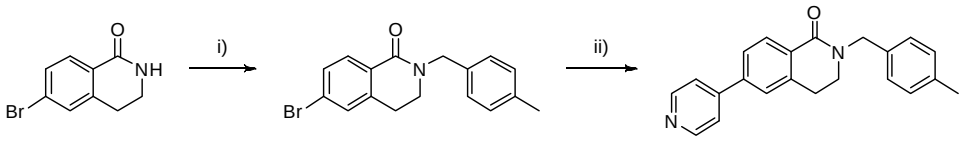
Figure 2. Overlay of **OX02983** (light blue) and **OX3699** (grey); blue spheres represent negative electrostatic field, red spheres represent positive electrostatic field, brown spheres represent hydrophobicity and small yellow sphere represent the van der Waals force.

DBQs have been investigated quite extensively in medicinal chemistry; examples have been reported as antiviral agents through inhibition of HIV replication [31,32]. Other analogues were found to

inhibit WDR5 protein-protein interactions, leading to inhibition of cancer cell proliferation [33–35]. [3-5]

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

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



Scheme 2. Synthesis of **OX3699**: Reagents and conditions: i) 4-methylbenzyl bromide (2 eq.), NaH (1.5 eq.), DMF, rt, 16 h (96 %); iii) 4 v. 4-pyridyl-$B(OH)_2$ (1.1 eq.), $Pd(dppf)Cl_2$ (5 mol%), K_2CO_3 (3 eq.), 1,4-dioxane/H_2O (,4:1), 90 ^oC 18 h.

The DBQ bearing the 3-and 4-pyridyl substituents (**OX03699** and **OX04236**) were active in the motility assay and led to similar EC_{50}s to the best results from the DHB series (with EC_{50} values of 21 µM and 46 µM respectively). Unfortunately, as soon as we moved away from the simple pyridyl substituent, all activity in the motility assay was lost again. The 2-amino pyrid-5-yl, the best example of ring **D** in the DHB series, was surprisingly inactive (**OX04238** EC_{50} >100 µM *vs*. **OX04117** EC_{50} 26 µM). Similarly, the methyl imidazole and the thiazole-substituted analogues (**OX04237** and **OX4739** respectively), also exhibited no activity in the motility assay, in contrast to their DHB counterparts suggesting that SARs did not correlate between the DHB and DBQ series. As the best results from the DQB and the DHB series were largely similar, we felt that this alternative core was not going to enhance substantially the potency of the compounds.

Collectively, these data have improved our understanding or provided insights into the SARs of the DHB/DQB family of compounds. The structure of cycles **A** and **B** in **OX02983** were found to be critical to activity; variations of the toluyl group for ring **C** generally also led to inactive compounds. Some variations of cycle **D** were tolerated, and there appeared to be a preference for a basic site

281 within the substituent. However, although we were able to alter the structure resulting in loss of
282 activity, we were unable to improve, only retain, activity.
283 Apart from the representative compounds presented in Table 1, further similar analogues and all
284 synthetic precursors were prepared and tested (S2 File). Together it gave us a library of 47 compounds
285 that could then be used against different parasites to understand whether these compound series
286 showed broad-spectrum anthelmintic activity.
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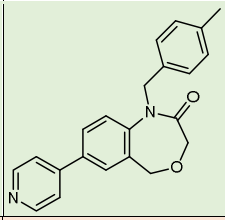
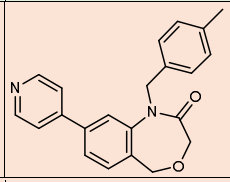
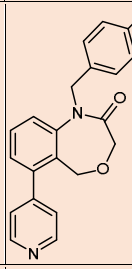
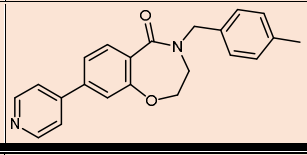
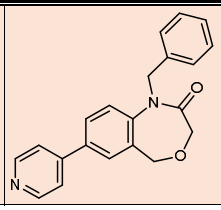
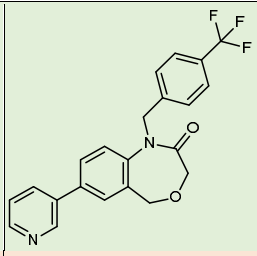
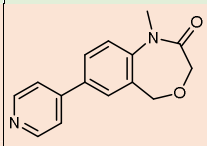
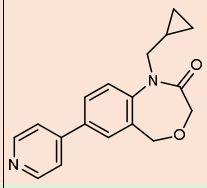
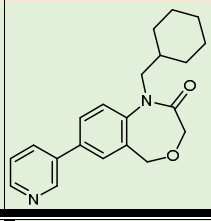
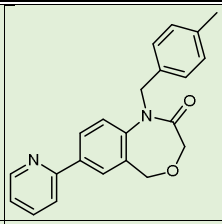
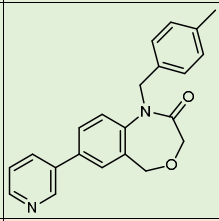
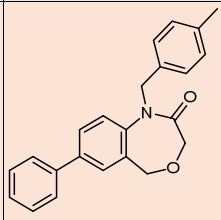
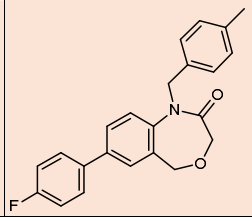
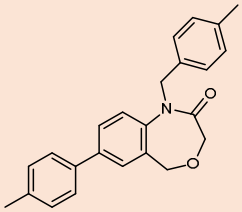
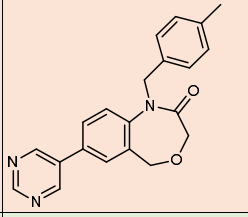
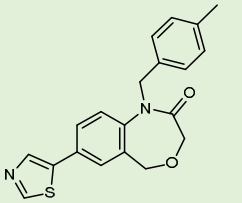
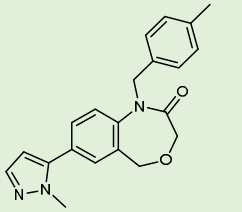
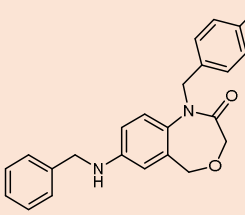
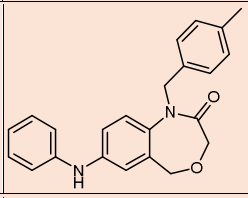
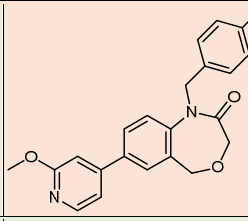
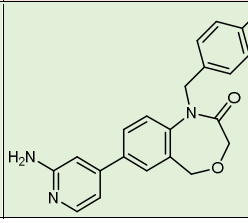
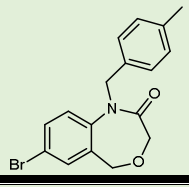
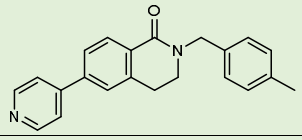
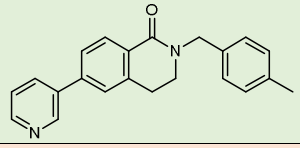
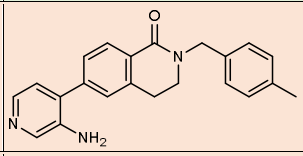
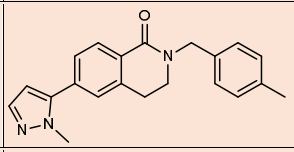
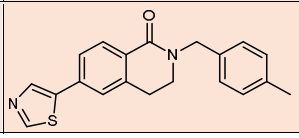
288

Compound	**Structure**	**EC_{50} (µM) in *T. muris* 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

289

290 **Table 1. Structures and EC_{50} of representative DHB compounds in the *T. muris* adult motility**
291 **assay.** All compounds investigated in this study are described in the S2 File. No activity means no
292 clear reduction in motility when tested at 100 μM. Where an EC_{50} estimate is shown, it was calculated
293 using a log-logistic model (n = 4) using the R package drc [28].

294

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

296 Whipworm is only one of many widely prevalent human helminth infections, and there are continuing
297 efforts to improve drug treatments for these diseases. There have been recent successes, such as the
298 approval of the veterinary medicine moxidectin for onchocerciasis [36], and the establishment of the
299 triple therapy albendazole, diethylcarbamazine citrate plus ivermectin as a macrofilaricidal treatment
300 for lymphatic filariasis suitable for mass drug administration [37,38]. However, sub-optimal efficacy,

problematic contraindications, and concerns that mass drug administration could lead to the spread of drug resistance, mean that repurposing of veterinary anthelmintics, improving drug combinations, and the development of new anthelmintics remain priorities [39–41].

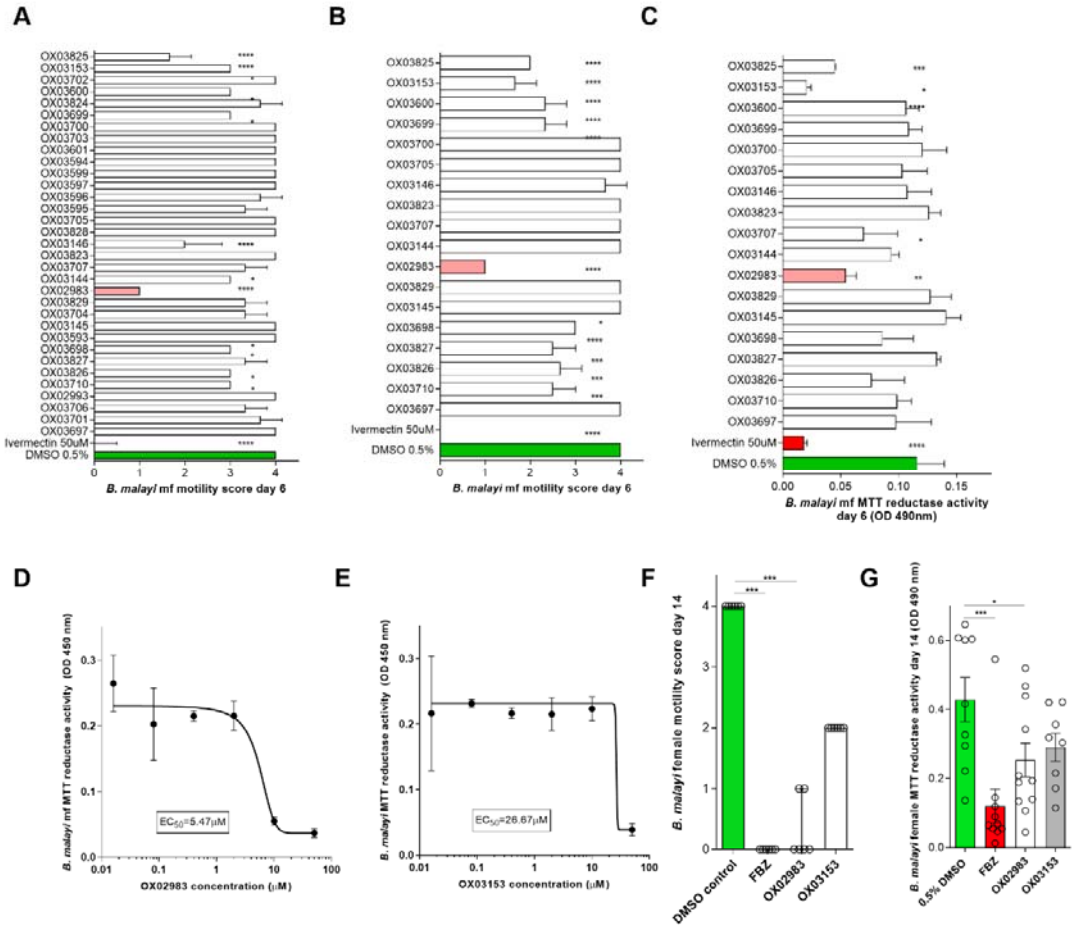
Development of a new anthelmintic is a long and expensive process, and funding for neglected tropical diseases is limited. Furthermore, multiple parasitic nematodes, for example the soil transmitted nematodes, *Ascaris*, *Trichuris* and the human hookworms, and the vector transmitted filarial nematodes,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

Activity against *B. malayi*

B. malayi is one of the tissue dwelling nematode parasites responsible for human lymphatic filariasis [42]. We first examined single dose efficacy of 33 DHB compounds at 10 µM against the *B. malayi* mf larval stage, with motility scored every 24 hours. The results after six days are shown in Fig 3A. Ivermectin was used as a positive control in the mf assay. OX02983 showed the most promise in this assay, reducing average motility to a score of 1. From this primary screen, 15 compounds that were determined to significantly impact *B. malayi* mf motility, plus an additional 5 with no discernible effect, were retested in a secondary screen (Fig 3B). These results confirmed the significant reduction in motility caused by 11 compounds, and confirmed the paralytic effect of OX02983. After six days drug exposure, mf were also tested for metabolic activity, a measure of parasite viability, using the MTT assay (Fig 3C). OX02983 and OX03153, in particular, showed activity in this assay, significantly reducing *B. malayi* mf MTT reductase activity on average by 53.4% and 81.9%, respectively (1-way ANOVA with Holm-Sidak's multiple comparison tests, $P<0.01$ and $P<0.0001$).. To determine the dose-dependent efficacy of OX02983 and OX03153, they were tested in a concentration response 6-day experiment (dose range 0.016-50µM) using MTT reductase activity as a

quantitative viability readout (Fig 3D,E). From this an EC_{50} concentration of 5.47 µM was determined for OX02983 and 26.67µM for OX03153.

Due to their efficacy against *B. malayi* mf, OX02983 and OX03153 were advanced for *in vitro* activity against adult *B. malayi*, utilising a novel, long-term adult worm lymphatic endothelial cell bilayer co-culture system. Adult female *B. malayi* exposed to vehicle control retained full survival and motility in culture over 14 days whereas the positive control, flubendazole (10µM) mediated complete paralytic activity by day 14 (Kruskal Wallis with Dunn's multiple comparisons tests, $P<0.001$) and significantly reduced metabolic activity by an average of 71.9% (1-way ANOVA with Holm-Sidak's multiple comparison tests, $P<0.001$) (Fig 3F-G). OX02983 (10µM) also mediated significant anti-filarial activities against adult *B. malayi* by day 14. Motility was completely hindered in 4/6 adult parasites by OX02983 (Kruskal Wallis with Dunn's multiple comparisons tests, $P<0.001$), whilst OX03153 mediated a 50% partial reduction in adult motility. OX02983 also significantly impacted on adult female *B. malayi* metabolic activity, on average by 40.8% (1-way ANOVA with Holm-Sidak's multiple comparison tests, $P<0.05$). Taken together, these results are encouraging because they show that compounds that are active against *T. muris* (a clade I nematode according to the phylogeny of Blaxter) are also active against evolutionarily-distant nematodes, as *B. malayi* is a clade III nematode [43].



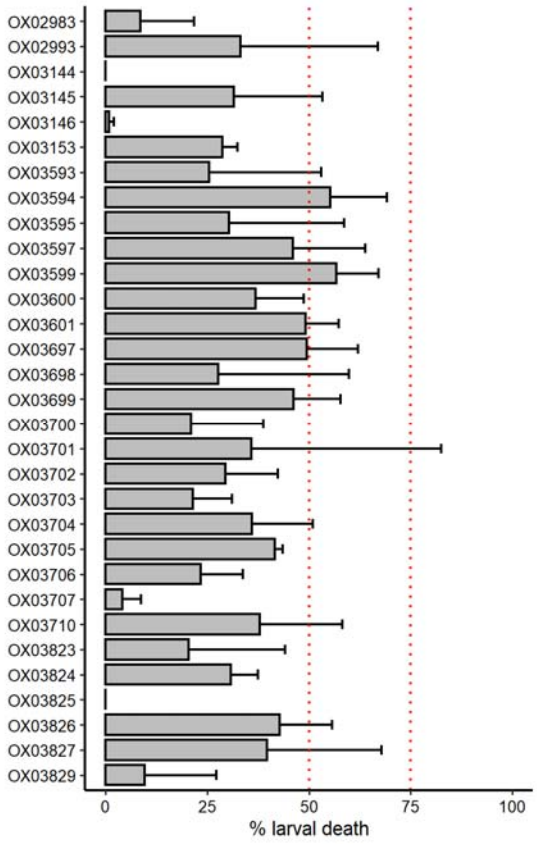
345

346 **Figure 3. Activity of 33 DHB compounds against *B. malayi* microfilariae and adults.** (A) Primary
347 screen – assessment of *B. malayi* mf motility (5 point scoring system) after six days continuous
348 exposure to 35 test compound screened at 10 µM in triplicate. Ivermectin (50 µM) was the positive
349 control. (B) Confirmatory mf motility and (C) metabolic activity screening of 15 active compounds
350 identified in (A) and five inactive compounds (10µM in triplicate). (D, E) 50% inhibitory
351 concentration (EC_{50}) assays of active compounds OX02983 and OX03153 on *B. malayi* microfilarial
352 metabolic activity after 6-day continuous exposure. Metabolic activity (C-E) was assessed by
353 colormetric MTT assay, data is optical density of mf extracts measured at 490nm. (F) Effects on adult
354 female *B. malayi* motility and (G) metabolic activity following 14 day continuous exposure to
355 OX02983 or OX03153 (10µM). Flubendazole (10µM) was used as a positive control in the assay.
356 Data plotted is mean ± SD of 3 replicates (A-E) median and range of 6 replicates (F) and mean ±

SEM of 9-11 replicates (G). Significant differences were determined by 1-way ANOVA with Holm-Sidak multiple comparisons test (A-C and G) or Kruskal-Wallis with Dunn's multiple comparisons test (F). Significance is indicated ****P<0.0001, ***P<0.001, **P<0.01 and *P<0.05.

Activity against *H. polygyrus*

H. polygyrus bakeri is an intestinal nematode parasite of laboratory mice [44]. It is a strongylid nematode, related to human hookworm species. 31 DHB compounds were tested at 100 µM against *ex vivo H. polygyrus* L3 stage worms (n = 2). The results are shown in Figure 4. The cut-off used to determine hits in this assay is 50% larval death [17]. Two compounds, OX03594 and OX03599, exceeded this level of larval death and were therefore considered active. They did not however reach the threshold for good activity (75%). Given the modest activity of these compounds against *H. polygyrus* we have not further pursued this direction at this point. Activity of DHB compounds against nematodes in three of the five clades of the phylum Nematoda, according to the phylogeny of Blaxter, supports the potential for development of a pan-nematode control agent from this compound series [43].



373

374 **Figure 4. Measurement of the activity of 31 DHB compounds against *H. polygyrus* L3 stage**
375 **worms.** Larval death is measured as the proportion of worms that respond to stimulus. Compounds
376 were tested in duplicate at 100 µM. Dashed lines indicates the cut-off (50%) used to determine hits in
377 this assay and the cut-off for good activity (75%) [17]: <50%, not active, 50-75% moderate activity,
378 >75% good and >90% excellent activity.

379

380

Discussion

Investigation of the DHB structure-activity relationship

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

This work has enabled us to define certain essential features of the anti-whipworm DHB compounds. The 4-pyridyl ring (cycle D in Fig 1) must be in the 2 position, unlike the analogues **OX03701** and **OX03707**. The amide moiety of the oxazepinone ring must be as in **OX02983**, and not as in **OX03704**. The oxazepinone nitrogen can be substituted with methylbenzyl, cyclohexyl and *p*-trifluromethylbenzyl (**OX02983**, **OX02993**, and **OX03144**), but not methyl, cyclopropyl, or benzyl groups. We also investigated in detail the replacement of cycle D. We found that removal of the oxygen from the DHB core was also consistent with similar activity to **OX02983** – the dihydrobenzoquinolinone compounds **OX03699** and **OX04236** had EC_{50} values of 21 and 42 µM respectively.

Targeting multiple helminth species with DHB family members

Despite being unable to improve efficacy against *Trichuris* substantially through structural modifications, we were able to demonstrate activity of our compounds against other helminth parasites. In drug discovery for NTDs, pan-anthelmintic activity is desirable given that polyparasitism in the target population is the norm. Thus, being able to target multiple species of helminths with a single drug administered via mass drug administration programmes is of significant benefit. Of particular note was the commonality in DHB compounds active against *T. muris* that were also active

against the tissue dwelling nematode parasite *B. malayi*. The ability of the DHB compounds to act against different clades within the nematode phylum is not unprecedented, indeed the co-administration of albendazole with ivermectin is currently advocated for control of *Trichuris*, and the same drug combination (in some situations supplemented with diethylcarbamazine) is widely used against lymphatic filariasis [38]. Indeed, the large-scale efforts to treat lymphatic filariasis have indirectly enhanced the number of people being treated for soil transmitted helminths [45]. Similarly, the alternative drug combination of albendazole and moxidectin is also being explored for the treatment of Trichuriasis given that moxidectin is an approved treatment for onchocerciasis [46].

Conclusions

In this study we have investigated the structure-activity relationship of the DHB compounds, defined essential features for anthelmintic action, and broadened the active series by the discovery of dihydrobenzoquinolinone compounds with activity against *T. muris* adult motility. We have also demonstrated that DHB and related compounds have activity against other parasitic nematodes: *B. malayi* and *H. polygyrus.* What we have not achieved however, is the substantive improvement in potency from the 20-50 µM range that would be desirable to progress this series with confidence to *in vivo* testing. Open science, where information is disclosed more freely than in traditional models, is proposed to accelerate and make more cost efficient drug discovery, especially in the context of neglected diseases [47,48]. We have therefore decided to report our progress at this point. We note that we do not yet know the target of the DHB/DBQ compounds in helminths. Identifying this target may facilitate the boost in activity we are striving for.

Acknowledgments

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Supporting information Captions

S1 File. Supporting information for synthetic chemistry

S2 File. Summary table of compound structures and assay results