

1 **2,4-Diaminothieno[3,2-d]pyrimidines, a new class of anthelmintic**  
2 **with activity against adult and egg stages of whipworm**

3 **Short title: Diaminothienopyrimidines, a new chemotype for the control of**  
4 **whipworm**

5 **Author List**

6 Frederick A. Partridge<sup>1¶</sup>, Ruth Forman<sup>2¶</sup>, Nicky J. Willis<sup>3¶</sup>, Carole J.R. Bataille<sup>3¶</sup>, Emma A. Murphy<sup>2-</sup>  
7 <sup>#a</sup>, Anwen E. Brown<sup>1</sup>, Narinder Heyer-Chauhan<sup>1</sup>, Bruno Marinič<sup>3</sup>, Daniel J.C. Sowood<sup>3</sup>, Graham M.  
8 Wynne<sup>3</sup>, Kathryn J. Else<sup>2\*</sup>, Angela J. Russell<sup>3,4\*</sup> and David B. Sattelle<sup>1\*</sup>

9 <sup>1</sup>Centre for Respiratory Biology, UCL Respiratory, Division of Medicine, University College London,  
10 London, United Kingdom

11 <sup>2</sup>Faculty of Biology, Medicine and Health, University of Manchester, Manchester, United Kingdom

12 <sup>3</sup>Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Oxford, United  
13 Kingdom

14 <sup>4</sup>Department of Pharmacology, University of Oxford, Oxford, United Kingdom

15 <sup>#a</sup>Current address: Liverpool School of Tropical Medicine, Liverpool, United Kingdom

16 \* Corresponding authors:

17 Email: [d.sattelle@ucl.ac.uk](mailto:d.sattelle@ucl.ac.uk) (DBS), [kathryn.else@manchester.ac.uk](mailto:kathryn.else@manchester.ac.uk) (KJE),  
18 [angela.russell@chem.ox.ac.uk](mailto:angela.russell@chem.ox.ac.uk) (AJR)

19 <sup>¶</sup>These authors contributed equally.

20

21 **Abstract**

22 The human whipworm *Trichuris trichiura* is a parasite that infects around 500 million people  
23 globally, with consequences including damage to physical growth and educational performance.  
24 Current drugs such as mebendazole have a notable lack of efficacy against whipworm, compared to  
25 other soil-transmitted helminths. Mass drug administration programs are therefore unlikely to achieve  
26 eradication and new treatments for trichuriasis are desperately needed. All current drug control  
27 strategies focus on post-infection eradication, targeting the parasite *in vivo*. Here we propose  
28 developing novel anthelmintics which target the egg stage of the parasite in the soil as an adjunct  
29 environmental strategy. As evidence in support of such an approach we describe the actions of a new  
30 class of anthelmintic compounds, the 2,4-diaminothieno[3,2-*d*]pyrimidines (DATPs). This compound  
31 class has found broad utility in medicinal chemistry, but has not previously been described as having  
32 anthelmintic activity. Importantly, these compounds show efficacy against not only the adult parasite,  
33 but also both the embryonated and unembryonated egg stages and thereby may enable a break in the  
34 parasite lifecycle.

## 35 **Author Summary**

36 The human whipworm, *Trichuris trichiura*, infects around 500 million people globally, impacting on  
37 their physical growth and educational performance. There are currently huge mass drug  
38 administration (MDA) programs aiming to control whipworm, along with the other major soil  
39 transmitted helminths, *Ascaris* and hookworm. However single doses of albendazole and  
40 mebendazole, which are used in MDA, have particularly poor effectiveness against whipworm, with  
41 cure rates less than 40%. This means that MDA may not be able to control and eliminate whipworm  
42 infection, and risks the spread of resistance to albendazole and mebendazole in the parasite  
43 population.

44 We are attempting to develop new treatments for parasitic worm infection, particularly focused on  
45 whipworm. We report the identification of a class of compounds, diaminothienopyrimidines  
46 (DATPs), which have not previously been described as anthelmintics. These compounds are effective  
47 against adult stages of whipworm, and also block the development of the model nematode *C. elegans*.

48 Our DATP compounds reduce the ability of treated eggs to successfully establish infection in a mouse  
49 model of human whipworm. These results support a potential environmental spray to control  
50 whipworm by targeting the infectious egg stage in environmental hotspots.

## 51 **Introduction**

### 52 **Current anthelmintics**

53 The benzimidazole anthelmintics albendazole and mebendazole are typically used to treat human  
54 whipworm infection but are compromised by lack of single-dose efficacy and the risk of resistance.  
55 Thus, existing drugs lack sufficient efficacy in mass drug administration (MDA) programs to  
56 adequately control or potentially eradicate whipworm. This is a major stumbling block in the WHO  
57 target to eliminate morbidity from soil transmitted helminthiases in children by 2020. The current  
58 approach for controlling soil-transmitted helminths such as *Trichuris* is mass drug administration of a  
59 single-dose of albendazole or mebendazole, typically repeated annually [1]. However for infection  
60 with *T. trichiura*, single doses of benzimidazoles lead to low cure rates, only 28% and 36% for  
61 albendazole and mebendazole respectively [2]. These cure rates are much lower than those of other  
62 major human soil-transmitted helminths, *Ascaris lumbricoides* and hookworm, demonstrating the  
63 need for improvements to therapy specifically targeting *Trichuris*. Indeed modelling studies have  
64 demonstrated that, due to these low cure rates, MDA with benzimidazoles does not interrupt  
65 whipworm transmission and thus cannot achieve eradication in many settings [3].

66 Furthermore, the experience from studies on veterinary parasites is that widespread usage of  
67 anthelmintics can lead to rapid development of resistance. The discovery of isolates of two species of  
68 gastrointestinal nematodes resistant to monepantel only four years after its introduction [4] underlies  
69 the real threat to control programmes imposed by emerging drug resistance. Indeed, the combination  
70 of MDA programs and low single-dose cure rates may facilitate the development of drug resistance in  
71 populations of human parasites. For example, resistance to benzimidazole drugs is caused by point  
72 mutations in  $\beta$ -tubulin. Such resistance mutations have been found in *T. trichiura* after mass drug  
73 administration [5], and have been found to increase in frequency after MDA. High frequency of  
74 resistance mutations in a population may be associated with lower egg-reduction rates after MDA [6].  
75 Whilst there is no clear evidence yet of widespread anthelmintic resistance in human populations,

76 identification of new drugs with novel mechanisms of actions is warranted to slow the development of  
77 drug resistance.

#### 78 *Trichuris* lifecycle

79 *A. T. trichiura* infection becomes patent when adult female worms, embedded in the gut of the host,  
80 start to lay eggs. A single female worm can lay up to 20,000 eggs per day and these unembryonated  
81 eggs pass out with the faeces and embryonate in the soil. Development only proceeds further if the  
82 embryonated eggs are accidentally consumed via contact of the next host with contaminated food,  
83 water or soil. Once ingested, signals for hatching are received when the eggs reach the large intestine  
84 [7,8], the newly emerged first stage larvae invade the mucosal epithelium and development to the  
85 adult stage of the parasite occurs through a succession of larval moults. Importantly, even when active  
86 infections are successfully treated, hosts are constantly re-infected due to high levels of infective eggs  
87 present within the water and soil, which can remain viable for years.

88 Current anthelmintic programmes, including those targeting *Trichuris*, focus on post-infection  
89 eradication of existing infections. However, lifecycle stages outside of the host are also potential  
90 viable targets for small molecule drugs. Thus, both preventing egg embryonation and reducing the  
91 infectivity of embryonated eggs prior to ingestion offer targets that would break the parasite lifecycle.

#### 92 Screening *ex vivo* *T. muris* adults for new anthelmintic chemotypes

93 The mouse whipworm, *T. muris*, is a convenient model of the human whipworm as it can be grown  
94 routinely in the laboratory via infection of severe combined immune deficiency (SCID) mice.  
95 Screening *ex vivo* adult *T. muris* has been used to test the anthelmintic activity of a variety of  
96 compounds, including approved drugs with the potential for repurposing, and also plant extracts [9–  
97 11]. We recently reported a small molecule screen utilising an automated assay for assessment of the  
98 motility of *ex vivo* *T. muris* adults. This screen led to the identification of a class of molecules termed  
99 dihydrobenzoxazepinone (DHB) which demonstrated encouraging activity in this assay, as well as the  
100 ability to reduce *in vivo* infectivity of treated eggs [12]. Most of the active molecules identified from

101 that screen belonged to the dihydrobenz[*e*][1,4]oxazepin-2(3*H*)-one chemotype, but interestingly one  
102 additional active was from a completely different structural class. Here we report the identification,  
103 synthesis and characterisation of a series of compounds belonging to this second chemotype, which  
104 has not previously been described as having anthelmintic activity, the 2,4-diamino  
105 thieno[3,2-*d*]pyrimidines (henceforth called diaminothienopyrimidines or DATPs).

## 106 **Materials and methods**

### 107 **Ethics statement**

108 All animal experiments were approved by the University of Manchester Animal Welfare and Ethical  
109 Review Board and performed under the regulation of the Home Office Scientific Procedures Act  
110 (1986) and the Home Office project licence 70/8127.

### 111 *In vivo* culture of *Trichuris muris*

112 *T. muris* worms were cultured using severe combined immune deficiency (SCID) mice, at the  
113 Biological Services Facility at the University of Manchester. Male and female mice were infected  
114 with 200 infective embryonated *T. muris* eggs via oral gavage. Thirty-five days later, the mice were  
115 sacrificed. Adult *T. muris* were obtained from the intestine as previously described [12]. Worms were  
116 maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with penicillin  
117 (500 U/mL) and streptomycin (500 µg/mL) at approximately 37 °C and studied on the same day.

### 118 *Ex vivo* *T. muris* adult maintenance for motility screen

119 Individual adult worms were added to wells containing 75 µL of RPMI-1640 medium, penicillin  
120 (500 U/mL), streptomycin (500 µg/mL) plus 1% v/v final concentration of dimethylsulfoxide  
121 (DMSO) or compound dissolved in DMSO. Plates were incubated at 37 °C, 5% CO<sub>2</sub>. Motility was  
122 determined after 24 hours.

### 123 Automated motility assay

124 An automated system was used to quantify worm movement. An earlier version of this system has  
125 been previously described [13,14]. Two hundred frame movies of the whole plate were recorded at 10  
126 frames per second and then motility determined by an algorithm based on thresholding pixel variance  
127 over time [15]. For the hit confirmation and expansion assays, library material was used at a final  
128 concentration of 100µM. Dose-response curves were calculated with the four factor log-logistic  
129 model using the R package *drc* [16] or using GraphPad Prism.





131 Chemical synthesis

132 Thin layer chromatography (TLC) was performed on aluminium sheets coated with 60 F<sub>254</sub> silica. All  
133 solvents are used anhydrous unless stated otherwise. NMR spectra were recorded on Bruker AV400  
134 (400 MHz), Bruker AVII 500 (500 MHz) or AVIIIHD 600 (600 MHz) instruments in the deuterated  
135 solvent stated. All chemical shifts ( $\delta$ ) are quoted in ppm and coupling constants ( $J$ ), which are not  
136 averaged, in Hz. Residual signals from the solvents were used as an internal reference using the stated  
137 deuterated solvent. Infrared spectra were recorded on a Perkin-Elmer 1750 IR Fourier Transform  
138 spectrophotometer using thin films on a diamond ATR surface (thin film). Only the characteristic  
139 peaks are quoted. Melting points were determined using a Stanford Research Systems EZ-Melt. Low  
140 resolution mass spectra ( $m/z$ ) were recorded on an Agilent 6120 spectrometer and high resolution  
141 mass spectra (HRMS  $m/z$ ) on a Bruker microTOF mass analyzer using electrospray ionization (ESI).  
142 Compounds were synthesised from commercially available starting materials, and fully characterised  
143 by Infrared (IR) Spectroscopy, Mass Spectrometry (ESI-MS, HRMS-ESI) and Nuclear Magnetic  
144 Resonance (<sup>1</sup>H and <sup>13</sup>C NMR). Spectra supporting the synthesis of these compounds are provided in  
145 the S1 File.

146 ***2-Chloro-N-(2-(chlorophenoxy)ethyl)thieno[3,2-*d*]pyrimidin-4-amine (2a)***

147 To a 20 mL microwave vial containing 2,4-dichlorothieno[3,2-*d*]pyrimidine (1.50 g, 7.32 mmol, 1.0  
148 equiv.) in 1,4-dioxane (15 mL) at RT was added 2-(2-chlorophenoxy)ethylamine (1.26 g, 7.32 mmol,  
149 1.0 equiv.) and *N,N*-diisopropylethylamine (2.5 mL, 14.64 mmol, 2.0 equiv.) under an argon  
150 atmosphere. The vessel was sealed and the reaction heated at 80 °C for 3 hours. The mixture was  
151 cooled to RT, concentrated *in vacuo* and the crude residue was purified by flash column  
152 chromatography (silica gel) to afford the title compound as an off-white solid (1.59 g, 64%).

153 mp = 118–119 °C;  $R_f$  = 0.2;  $\nu_{\max}$  (film)/cm<sup>-1</sup> = 3398m (NH), 3088w (CH), 2970w (CH), 1586s  
154 (arom.), 1539m (arom.), 1483m (arom.); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  7.75 (1H, d,  $J$  = 5.4 Hz), 7.39  
155 (1H, dd,  $J$  = 8.0, 1.6 Hz), 7.36 (1H, d,  $J$  = 5.4 Hz), 7.23 (1H, ddd,  $J$  = 8.3, 7.5, 1.6 Hz), 7.02 (1H, dd,  $J$   
156 = 8.4, 1.4 Hz), 6.96 (1H, apparent td,  $J$  = 7.9, 1.3 Hz), 5.79 (1H, t,  $J$  = 5.4 Hz), 4.30 (2H, t,  $J$  = 5.0 Hz)

157 4.13 (2H, apparent q,  $J = 5.4$  Hz);  $^{13}\text{C}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  161.4, 158.1, 157.6, 153.9, 132.6,  
158 130.5, 128.1, 124.9, 123.5, 122.6, 114.7, 114.3, 68.1, 40.5; LRMS (ESI) calculated for  
159  $[\text{C}_{14}\text{H}_{11}\text{ON}_3^{35}\text{Cl}_2^{32}\text{S}-\text{H}]^- = 338.0$ , found 337.9,  $[\text{M}-\text{H}]^-$ , 100%, calculated for  $[\text{C}_{14}\text{H}_{11}\text{ON}_3^{35}\text{Cl}^{37}\text{Cl}^{32}\text{S}-$   
160  $\text{H}]^- = 340.0$ , found, 339.9  $[\text{M}-\text{H}]^-$ , 60%; HRMS (ESI $^+$ ) calculated for  $[\text{C}_{14}\text{H}_{11}\text{ON}_3^{35}\text{Cl}_2^{32}\text{S}+\text{H}]^+ =$   
161 340.0073, found 340.0071,  $[\text{M}+\text{H}]^+$ .

## 162 **2-Chloro-*N*-(2-phenoxyethyl)thieno[3,2-*d*]pyrimidin-4-amine (2b)**

163 To a 20 mL microwave vial containing 2,4-dichlorothieno[3,2-*d*]pyrimidine (1.0 g, 5.0 mmol, 1.0  
164 equiv.) in 1,4-dioxane (10 mL) at RT was added 2-phenoxyethylamine (0.6 mL, 5.0 mmol, 1.0 eq.)  
165 and *N,N*-diisopropylethylamine (1.7 mL, 10.0 mmol, 2.0 equiv.) under an argon atmosphere. The  
166 vessel was sealed and the reaction heated at 80 °C for 3 hours. The mixture was cooled to RT,  
167 concentrated *in vacuo* and the crude residue was purified by flash column chromatography (silica gel)  
168 to afford the title compound as an off-white solid (1.23 g, 80%).

169

170 mp = 115.5–116.9 °C;  $R_f = 0.5$  (EtOAc: Petroleum; 1:4);  $\nu_{\text{max}}$  (film)/ $\text{cm}^{-1} = 3228\text{w}$  (NH), 3041w  
171 (CH), 2962w (CH), 1597s (arom.), 1581s (arom.), 1533m (arom.), 1511m (arom.), (arom.), 1496m  
172 (arom.), 1469m (arom.), 1434m (arom.);  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.72 (1H, d,  $J = 5.4$  Hz), 7.34  
173 (1H, d,  $J = 5.4$  Hz), 7.32-7.28 (2H, m), 6.97 (1H, app t,  $J = 7.3$  Hz), 6.96 -6.92 (2H, m), 5.78 (1H, t,  $J$   
174 = 4.8 Hz), 4.22 (2H, t,  $J = 5.1$  Hz), 4.11-4.06 (2H, m);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  161.4, 158.4,  
175 158.1, 157.5, 132.6, 129.7, 124.8, 121.5, 114.6, 114.1, 66.2, 40.9; LRMS (ESI $^+$ ) calculated for  
176  $[\text{C}_{14}\text{H}_{12}\text{ON}_3^{35}\text{Cl}_2^{32}\text{S}+\text{H}]^+ = 306.0$ , found 306.0,  $[\text{M}+\text{H}]^+$ , 100%, calculated for  
177  $[\text{C}_{14}\text{H}_{12}\text{ON}_3^{35}\text{Cl}^{37}\text{Cl}^{32}\text{S}+\text{H}]^+ = 308.0$ , found 308.0,  $[\text{M}+\text{H}]^+$ , 40%, calculated for  
178  $[\text{C}_{14}\text{H}_{12}\text{ON}_3^{35}\text{Cl}_2^{32}\text{S}+\text{Na}]^+ = 328.0$ , found 328.0,  $[\text{M}+\text{H}]^+$ , 60%, calculated for  
179  $[\text{C}_{14}\text{H}_{12}\text{ON}_3^{35}\text{Cl}^{37}\text{Cl}^{32}\text{S}+\text{Na}]^+ = 330.0$ , found 330.0,  $[\text{M}+\text{H}]^+$ , 20%; HRMS (ESI $^+$ ) calculated for  
180  $[\text{C}_{14}\text{H}_{12}\text{ON}_3^{35}\text{Cl}_2^{32}\text{S}+\text{H}]^+ = 306.0462$ , found 306.0462,  $[\text{M}+\text{H}]^+$ .

181

## 182 **General Synthetic Procedure**

183 To a 10 mL microwave vial containing 2-chlorothieno[3,2-*d*]pyrimidine (1.0 equiv) in <sup>i</sup>PrOH (10  
184 μL/mg chloride) at room temperature was added the requisite amine (10.0 equiv.) under an argon  
185 atmosphere. The vessel was sealed and the mixture heated at 100°C for 16-24 hours. The reaction was  
186 cooled to ambient temperature (RT), concentrated *in vacuo* and the crude residue was purified by  
187 flash column chromatography (silica gel).

188

189 ***N*2-Methyl-*N*4-(2-phenoxyethyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (3a, OX02925)**

190 Following general procedure 1, the title compound was obtained from **2b** (600 mg, 1.96 mmol, 1.0  
191 equiv.) and methylamine (2.0 M in THF, 9.8 mL, 19.6 mmol, 10.0 eq). Purification by flash column  
192 chromatography (MeOH:CH<sub>2</sub>Cl<sub>2</sub>; 1:49 v/v) followed by trituration with cold Et<sub>2</sub>O afforded the desired  
193 product as a pale yellow viscous oil (526 mg, 89%).

194

195  $R_f = 0.2$  (MeOH:CH<sub>2</sub>Cl<sub>2</sub>; 1:49 v/v);  $v_{max}$  (film)/cm<sup>-1</sup> = 3418w (NH), 3232w (NH), 3038w (CH), 2936w  
196 (CH), 1585s (arom.), 1532s (arom.), 1508s (arom.), 1460s (arom.), 1405m (arom.); <sup>1</sup>H NMR (500  
197 MHz, CDCl<sub>3</sub>) δ 7.55 (1H, d, *J* = 5.4 Hz), 7.33-7.28 (2H, m), 7.15 (1H, d, *J* = 5.4 Hz), 7.00-6.96 (1H,  
198 m), 6.96-6.93 (2H, m), 5.16 (1H, brs), 4.83 (1H, brs), 4.21 (2H, t, *J* = 5.26 Hz), 4.02 (2H, m), 3.04  
199 (3H, d, *J* = 5.04 Hz); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 161.9, 161.8, 158.5, 157.4, 130.4, 129.6, 124.1,  
200 121.2, 114.5, 106.5, 66.6, 40.2, 28.7; LRMS (ESI<sup>+</sup>) calculated for [C<sub>15</sub>H<sub>16</sub>ON<sub>4</sub><sup>32</sup>S+H]<sup>+</sup> = 301.1, found  
201 301.1 [M+H]<sup>+</sup>, 100%; HRMS (ESI<sup>+</sup>) calculated for [C<sub>15</sub>H<sub>16</sub>ON<sub>4</sub>C<sup>32</sup>S+H]<sup>+</sup> = 301.1119, found 301.1118  
202 [M+H]<sup>+</sup>.

203

204 ***N*4-(2-(2-Chlorophenoxy)ethyl)-*N*2-(2-methoxyethyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (3b,  
205 OX02926)**

206 Following general procedure 1, the title compound was obtained from **2a** (600 mg, 1.76 mmol, 1.0  
207 equiv.) and 2-methoxyethylamine (1.5 mL, 17.6 mmol, 10.0 eq). Purification by flash column  
208 chromatography (MeOH:CH<sub>2</sub>Cl<sub>2</sub>; 1:49 v/v) followed by trituration with cold Et<sub>2</sub>O afforded the desired  
209 product as an off-white solid (380 mg, 57%).

210

211 mp = 69–97 °C (Et<sub>2</sub>O); R<sub>f</sub> = 0.1 (MeOH:CH<sub>2</sub>Cl<sub>2</sub>; 1:49 v/v); v<sub>max</sub> (film)/cm<sup>-1</sup> = 3424w (NH),  
212 3304w (NH), 3076w (CH), 2949w (CH), 1606m (arom.), 1532s (arom.), 1476m (arom.), 1460m  
213 (arom.), 1444m (arom.), 1412m (arom.); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.56 (1H, d, *J* = 5.3 Hz), 7.39  
214 (1H, dd, *J* = 7.9, 1.4 Hz), 7.23-7.21 (1H, m), 7.13 (1H, d, *J* = 5.3 Hz), 7.00-6.92 (2H, m), 5.29 (1H,  
215 s), 5.17 (1H, s) 4.27 (2H, t, *J* = 5.2 Hz), 4.05 (2H, apparent q, *J* = 5.4 Hz), 3.68-3.59 (4H, m), 3.40  
216 (3H, s); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ 161.8, 161.1, 157.3, 154.0, 130.5, 130.3, 127.8, 124.0, 123.3,  
217 122.1, 114.3, 106.8, 71.6, 68.2, 58.7, 41.4, 39.9; LRMS (ESI<sup>+</sup>) calculated for [C<sub>17</sub>H<sub>19</sub>O<sub>2</sub>N<sub>4</sub><sup>35</sup>Cl<sup>32</sup>S+H]<sup>+</sup>  
218 = 379.1, found 379.1, [M+H]<sup>+</sup>, 100%, calculated for [C<sub>17</sub>H<sub>19</sub>O<sub>2</sub>N<sub>4</sub><sup>35</sup>Cl<sup>32</sup>S+Na]<sup>+</sup> = 401.1, found 401.1,  
219 [M+Na]<sup>+</sup>, 10%; HRMS (ESI<sup>+</sup>) calculated for [C<sub>17</sub>H<sub>19</sub>O<sub>2</sub>N<sub>4</sub><sup>35</sup>Cl<sup>32</sup>S+H]<sup>+</sup> = [M+H]<sup>+</sup>, 379.0990, found  
220 379.0991 [M+H]<sup>+</sup>.

221

222 *N4-(2-(2-chlorophenoxy)ethyl)-N2-(2-methoxybenzyl)thieno[3,2-d]pyrimidine-2,4-diamine*

223 **(3c, OX03143)**

224 Following general procedure 1, the title compound was obtained from **2a** (240 mg, 0.70 mmol, 1.0  
225 eq.) and 2-methoxybenzylamine (0.92 mL, 7.0 mmol, 10.0 eq.). Purification by flash column  
226 chromatography (MeOH:CH<sub>2</sub>Cl<sub>2</sub>; 3:37 v/v) afforded the desired product (189 mg, 61%) as a thick pale  
227 yellow oil.

228

229 R<sub>f</sub> = 0.4 (MeOH:CH<sub>2</sub>Cl<sub>2</sub>; 3:22 v/v); v<sub>max</sub> (film)/cm<sup>-1</sup> = 3424w (NH), 3247w (NH), 2935w (CH), 1587m  
230 (arom.), 1553 (arom.), 1487 (arom.), 1461 (arom.); <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 7.54 (1H, d, *J* = 5.3  
231 Hz), 7.38 (1H, dd, *J* = 8.1, 1.7 Hz), 7.36 (1H, s) 7.22 (1H, ddd, *J* = 9.5, 7.5, 1.6 Hz), 7.20 (1H, ddd, *J*  
232 = 8.2, 7.5, 1.6 Hz), 7.12 (1H, d, *J* = 5.3 Hz), 6.93 (1H, dt, *J* = 7.5, 1.5 Hz), 6.91 (1H, ddd, *J* = 8.1, 7.1,  
233 1.1 Hz), 6.89 (2H, d, *J* = 7.9 Hz), 5.34 (1H, br), 5.29 (1H, t, *J* = 5.3 Hz), 4.68 (2H, d, *J* = 6.2 Hz) 4.20  
234 (2H, t, *J* = 5.3 Hz), 4.04 (2H, apparent q, *J* = 5.3 Hz), 3.87 (3H, s); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ  
235 161.9, 161.2, 157.6, 157.4, 154.1, 130.5, 130.3, 129.1, 128.1 (x2), 127.8, 123.9, 123.3, 122.1, 120.3,  
236 114.3, 110.1, 106.6, 68.2, 55.3, 41.4, 40.0; LRMS (ESI<sup>+</sup>) calculated for [C<sub>22</sub>H<sub>21</sub>O<sub>2</sub>N<sub>4</sub><sup>35</sup>Cl<sup>32</sup>S+H]<sup>+</sup> =  
237 441.1, found 441.2, [M+H]<sup>+</sup>, 100%; HRMS (ESI<sup>+</sup>) calculated for [C<sub>22</sub>H<sub>21</sub>O<sub>2</sub>N<sub>4</sub><sup>35</sup>Cl<sup>32</sup>S+H]<sup>+</sup> = 441.1147,  
238 found 441.1142 [M+H]<sup>+</sup>.

239

240 ***N4-(2-(2-chlorophenoxy)ethyl)-N2-methylthieno[3,2-d]pyrimidine-2,4-diamine (3d, OX03147)***

241 Following general procedure 1, the title compound was obtained from **2a** (237 mg, 0.70 mmol) and  
242 methylamine (2.0 M in THF) (3.5 mL, 7.0 mmol, 10 eq). Purification by flash column  
243 chromatography (MeOH:CH<sub>2</sub>Cl<sub>2</sub>; 1:19 v/v) afforded the desired product (218 mg, 93%) as a pale  
244 brown oil.

245  $R_f = 0.4$  (MeOH:CH<sub>2</sub>Cl<sub>2</sub>; 1:19 v/v);  $v_{max}$  (film)/cm<sup>-1</sup> = 3247w (NH), 2940w (CH), 1588m (arom.),  
246 1552m (arom.), 1510m (arom.), 1484m (arom.), 1461m (arom.), 1446m (arom.), 1406 (arom.); <sup>1</sup>H  
247 NMR (600 MHz, CDCl<sub>3</sub>)  $\delta$  7.56 (1H, d,  $J = 5.3$  Hz), 7.38 (1H, dd,  $J = 7.9, 1.7$  Hz) 7.21 (1H, ddd,  $J =$   
248 8.2, 7.5, 1.7 Hz), 7.15 (1H, d,  $J = 5.3$  Hz), 6.98 (1H, dd,  $J = 8.2, 1.5$  Hz), 6.95 (1H, dd,  $J = 7.5, 1.5$   
249 Hz) 5.31 (1H, t,  $J = 5.5$  Hz), 4.84 (1H, s) 4.27 (2H, t,  $J = 5.4$  Hz), 4.06 (2H, apparent q,  $J = 5.5$  Hz),  
250 3.04 (3H, d,  $J = 5.0$  Hz); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>)  $\delta$  162.0, 161.9, 157.5, 154.2, 130.7, 130.5,  
251 128.0, 124.2, 123.6, 122.3, 114.6, 106.9, 68.5, 40.1, 28.9; LRMS (ESI<sup>+</sup>) calculated for  
252 [C<sub>15</sub>H<sub>15</sub>ON<sub>4</sub><sup>35</sup>Cl<sup>32</sup>S+H]<sup>+</sup> = 335.1, found 335.0, [M+H]<sup>+</sup>, 100%, calculated for [C<sub>15</sub>H<sub>15</sub>ON<sub>4</sub><sup>37</sup>Cl<sup>32</sup>S+H]<sup>+</sup>  
253 = 337.1, found 337.0, [M+H]<sup>+</sup>, 30%, HRMS (ESI<sup>+</sup>) calculated for [C<sub>15</sub>H<sub>15</sub>ON<sub>4</sub><sup>35</sup>Cl<sup>32</sup>S+H]<sup>+</sup> =  
254 335.0728, found 335.0725 [M+H]<sup>+</sup>, calculated for [C<sub>15</sub>H<sub>15</sub>ON<sub>4</sub><sup>37</sup>Cl<sup>32</sup>S+H]<sup>+</sup> = 337.0698, found  
255 337.0695 [M+H]<sup>+</sup>.

256 ***C. elegans* growth assay**

257 A mixed-stage *C. elegans* N2 population was obtained by liquid culture (20 °C) according to standard  
258 methods [17]. It was then bleached to obtain an egg population with 1.5 mL 4M NaOH, 2.4 mL  
259 NaOCl, 2.1 mL water, washed three times, and allowed to hatch in 50 mL S-basal buffer at 20 °C  
260 overnight to obtain a synchronised L1 population. For the growth assay, 49  $\mu$ L of S-complete buffer  
261 and 1  $\mu$ L of DMSO or DMSO plus compound were added to each well of 96-well plates. 50  $\mu$ L of a  
262 worm suspension (approximately 20 synchronised L1 worms, 1% w/v *E. coli* HB101 in S-complete  
263 buffer) were then added to each well. Plates were incubated at 20 °C before imaging 5 days later.  
264 Worm movement was stimulated by inserting and removing a 96-well PCR plate into/from the wells  
265 of the assay plate, and then whole plate 200 frame movies were recorded at 30 frames per second.

266 Growth was quantified as a correlate of movement using the same automated system described earlier,  
267 which estimates movement for each well by categorising pixels as imaging movement if their  
268 variance is greater the mean plus one standard deviation of the variances of all the pixels on the plate  
269 [15].

#### 270 Cytotoxicity testing

271 The mouse rectal epithelial cell line CMT-93 (LGC Promochem, Teddington, United Kingdom) was  
272 used for these studies. The WST-8 and neutral red cytotoxicity assays were performed as described  
273 [12]. Briefly, cells were cultured with test compounds, chlorpromazine positive control or DMSO  
274 alone (final compound concentrations of 0 to 100  $\mu$ M) for 72 hours. The WST-8 assay was then  
275 carried out using the Cell Counting Kit – 8 (Sigma Aldrich # 96992) with an incubation time of 2  
276 hours. This time was chosen according to the manufacturer's instructions and was such that the  
277 absorbance of the WST-8 formazan dye was within the linear range of the microplate reader.  
278 Following this assay, the medium was exchanged, and the ability of the cells to take up the dye  
279 neutral red (concentration 33  $\mu$ g/mL, incubation time 2 hours) was determined using a microplate  
280 reader (absorbance at 540 nm). Results were analysed using GraphPad Prism and fitted using a log-  
281 logistic model.

#### 282 *In vitro* and *in vivo* establishment of infection

283 100 infective embryonated eggs were incubated in deionised water with 1% v/v DMSO or test  
284 compounds at a final concentration of 100  $\mu$ M in 1% v/v DMSO for 14 days at room temperature in  
285 the dark. Eggs were then washed and resuspended in deionised water. For *in vitro* hatching assays 100  
286 eggs were added to 1 mL of *E. coli* bacterial culture grown in LB broth overnight at 37 °C shaking at  
287 200 rpm. Egg-bacterial cultures were incubated for 24 hours at 37 °C, 5% CO<sub>2</sub> and hatching  
288 determined following blinding by visual examination under a dissecting microscope. For *in vivo*  
289 hatching assays, 40 eggs were counted under a dissecting microscope and given to a SCID mouse in  
290 200  $\mu$ L water. At day 15 post-infection mice were culled and the number of L2 larvae present in the  
291 caecae and colon enumerated in a blinded manner under a dissecting microscope.

292 Statistical analysis of *in vivo* establishment of infection data

293 The experiment was conducted in two ‘experimental batches’. For batch one there were 5 mice in  
294 each of the DMSO and **OX02926** groups. For batch two there were 9 mice in each of the DMSO and  
295 **OX02926** groups. The raw data (number of worms that established infection in each mouse) are  
296 shown separated by batch and treatment in the S2 Figure.

297 To analyse the data we used a two-way ANOVA (worm number ~ treatment \* batch). This showed a  
298 significant effect of treatment [ $F(1,24) = 8.520$ ,  $P = 0.00752$ ]. It also showed a significant effect of  
299 batch [ $F(1,24) = 10.956$ ,  $P = 0.00294$ ]. There was no significant interaction between treatment and  
300 batch [ $F(1,24) = 0.296$ ,  $P = 0.59153$ ]. The significant effect of batch reflected that in both DMSO-  
301 and **OX02926**-treated groups, the number of worms that established infection was generally lower in  
302 mouse batch 1 than in batch 2 (S2 Figure). Variation in control worm establishment, which is  
303 commonplace in *Trichuris* infections due to natural variation in egg infectivity from a standardised  
304 egg number, was within expected ranges. We therefore took the approach of normalising each data  
305 point by dividing by the mean of the DMSO-treated group for that batch. This yielded the % batch  
306 normalised infection establishment.

307 We used a two-way ANOVA (% batch normalised infection establishment ~ treatment \* batch) to  
308 analyse the data. There was a significant effect of treatment [ $F(1,24) = 9.569$ ,  $P = 0.00497$ ] but no  
309 effect of batch [ $F(1,24) = 0.083$ ,  $P = 0.77618$ ] or interaction [ $F(1,24) = 0.083$ ,  $P = 0.77618$ ]. We therefore  
310 conducted a post-hoc Tukey HSD test which showed that infection establishment in the **OX02926**-  
311 treated group was significantly different from the DMSO-treated control group ( $P = 0.0050$ ).

312 Embryonation assay

313 One hundred unembryonated eggs were treated with water, 1% v/v DMSO in water or test compounds  
314 at a final concentration of 100  $\mu$ M (unless stated) with 1% v/v DMSO, in the dark at 26 °C, either for  
315 56 days or for shorter periods as described. Images were collected on an Olympus BX63 upright  
316 microscope using a 60x / 1.42 PlanApo N (Oil) objective and captured and white-balanced using

317 an DP80 camera (Olympus) in monochrome mode through CellSens Dimension v1.16 (Olympus).

318 Images were then processed and analysed using the image analysis platform Fiji [18].

319 **Data availability**

320 Structures of resynthesized compounds have been deposited in the PubChem database with CID

321 49790760, 49790669, 46948320 and 49778268 and SID 348479445, 348479446, 348479447 and

322 348479448. Assay results for resynthesized compounds have been deposited in the PubChem database

323 with assay ID 1259352 and 1259353.

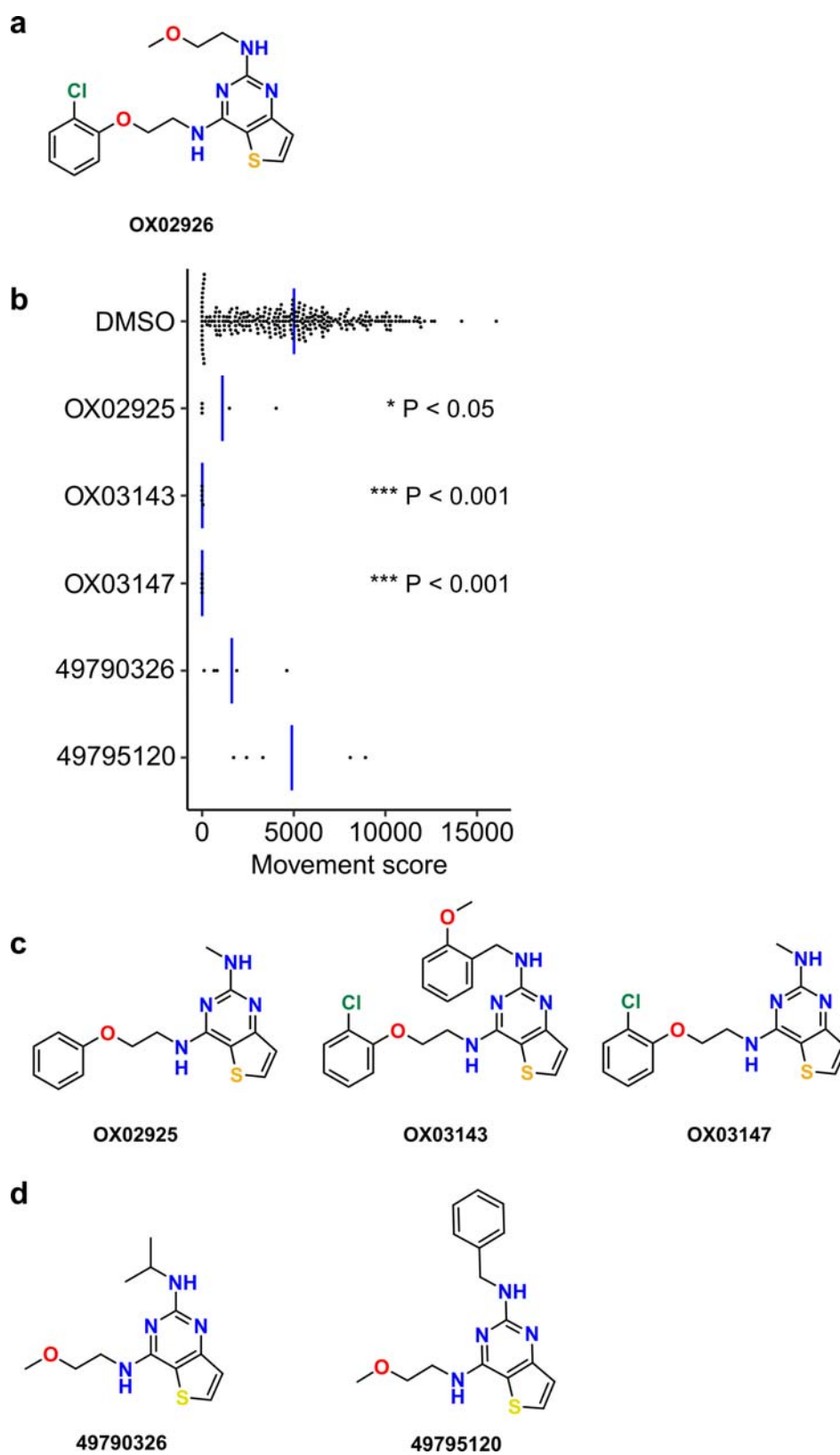


## 324 **Results**

### 325 *Ex vivo T. muris* adult motility screen

326 We have recently described a small molecule screen for new anthelmintics, which used reduction or  
327 loss of motility of adult *ex vivo T. muris* as an endpoint for screening [12]. This screen was designed  
328 to identify compounds active on *Trichuris* as existing drugs are notably less efficacious against this  
329 nematode, and it is comparatively evolutionarily distant to nematodes typically screened in  
330 anthelmintic-discovery efforts, such as *H. contortus*, *M. incognita* and *C. elegans*. From this primary  
331 screen, we found 13 members of the dihydrobenzoxazepinone chemotype, which had not previously  
332 been shown to have anthelmintic activity.

333 In this report we describe the identification of a second new anthelmintic chemotype from this screen.  
334 A single 2,4-diaminothieno[3,2-*d*]pyrimidine (DATP) compound was found in the primary screen.  
335 This has been given the identifier **OX02926** (Fig 1a). We confirmed this activity in a secondary  
336 screen using the same source sample (DMSO solution containing 10 mM compound), and also tested  
337 a number of structurally-related compounds from our small molecule collection using the same assay  
338 (Fig 1b). The rationale for this was to gain greater confidence in the screening hit and also to explore  
339 the activity of “near-neighbour” molecules with the same core 2,4-diaminothieno[3,2-*d*]pyrimidine  
340 structure, which could support the early development of the series. The hit expansion process led to  
341 the identification of three further active molecules in this series **OX02925**, **OX03143** and **OX03147**  
342 (Fig 1c). Two structurally-related compounds were however not active in this assay (Fig 1d).

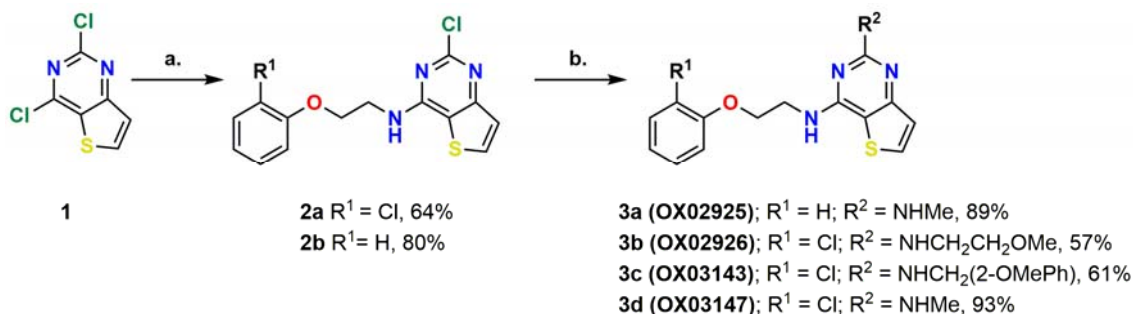


343

344 **Fig 1. Identification of a diaminothienopyrimidine series from an *ex vivo* *T. muris* motility**  
345 **screen. (a)** Structure of the hit compound, which was given the identifier OX02926. **(b)** Hit expansion  
346 by testing of structurally-related compounds using library material, assay concentration 100µM.  
347 Significance was determined by a two-sided Mann-Whitney test compared to DMSO-only controls,  
348 adjusted for multiple comparisons using the Bonferroni method (for test compounds n=5, each  
349 replicate on different assay plates, each point indicates one assay well). Blue bar indicates mean  
350 movement score. **(c)** Structures and identifiers of additional active compounds from this class. **(d)**  
351 Structures and PubChem CID accession numbers for the two compounds that were not significantly  
352 active in this assay.

### 353 Resynthesis of active compounds

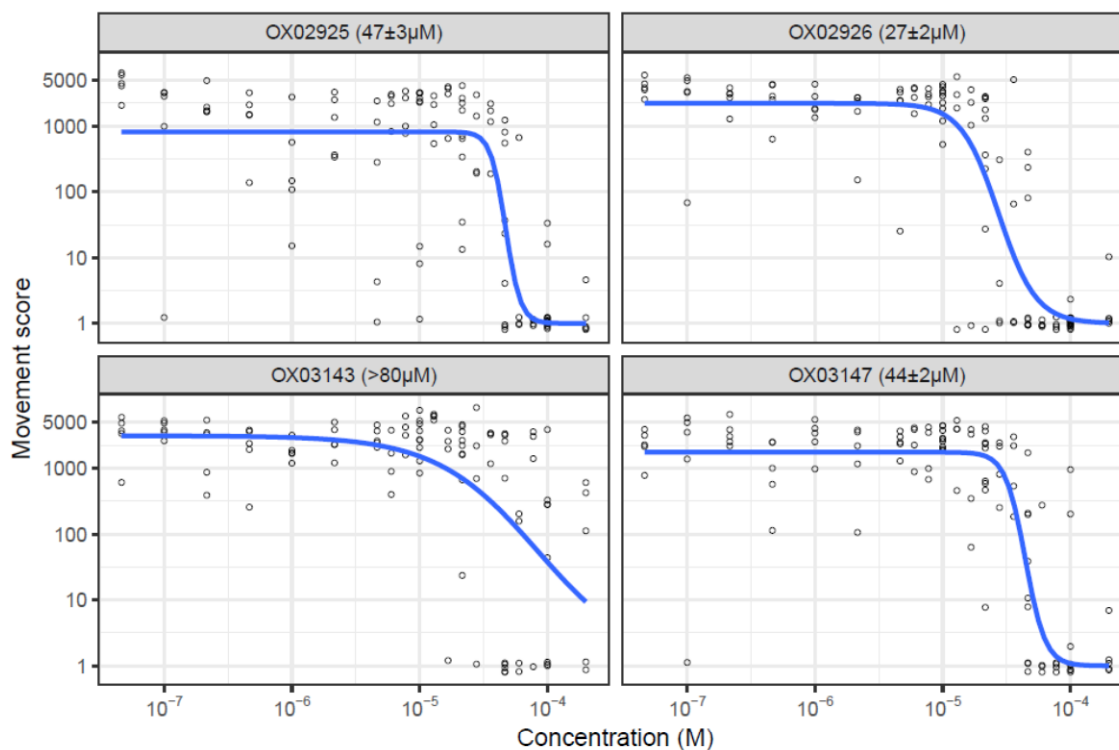
354 Having identified promising active DATPs from testing of DMSO solution samples of compounds,  
355 these were then resynthesised to obtain authentic, unambiguously characterised samples from which  
356 confirmatory screening could take place. Compound resynthesis is important since DMSO solution  
357 samples can degrade over time, and this often leads to so-called ‘false positive’ hits [19]. These  
358 compounds could be readily prepared in two steps from commercially available 2,4-  
359 dichlorothieno[3,2-*d*]pyrimidine **1**, via two sequential nucleophilic aromatic substitution reactions.  
360 Treatment of **1** with 2-(2-chlorophenoxy)ethylamine or 2-phenoxyethylamine gave exclusively  
361 monosubstitution affording **2a** and **2b** as a single regioisomer in 64% and 80% yield respectively.  
362 Subsequent displacement reaction at C4 gave authentic samples of **OX02925**, **OX02926**, **OX03143**  
363 and **OX03147** in 57 – 91% yield (Fig 2).



364

365 **Fig 2. Synthetic route to putative hit compounds** (a) Substituted 2-phenoxyethan-1-amine  
366 (1.0 equiv.), DIPEA (2.0 equiv.), 1,4-dioxane, 80 °C, 3 hours. (b) Alkyl amine (10.0 equiv.), <sup>t</sup>PrOH,  
367 100 °C, 16-24 hours.

368 Activity of resynthesised diaminothienopyrimidines in the *T. muris ex vivo* adult motility assay  
369 The resynthesized hits were then tested in this screen and a concentration-response curve constructed,  
370 thereby confirming the anthelmintic activity of several examples of this structural class. (Fig 3,  
371 Table 1).



373 **Fig 3. Concentration-response curves for resynthesized DATPs in the *T. muris ex vivo* adult**  
374 **motility assay.** n=4 or 5 wells per concentration per compound, each replicate on a different 96-well  
375 plate using worms from different mice. Blue line indicates concentration-response curve fitted with  
376 the 3-factor log-logistic model using *drc* [16]. Figure in parenthesis indicates EC<sub>50</sub> estimate ± standard  
377 error from this model. OX03143 did not clearly form a sigmoidal curve in the range of concentrations  
378 used in this assay so we report the EC<sub>50</sub> estimate as > 80µM.

379

380 Chemical properties of the hit series and synthetic suitability for further development

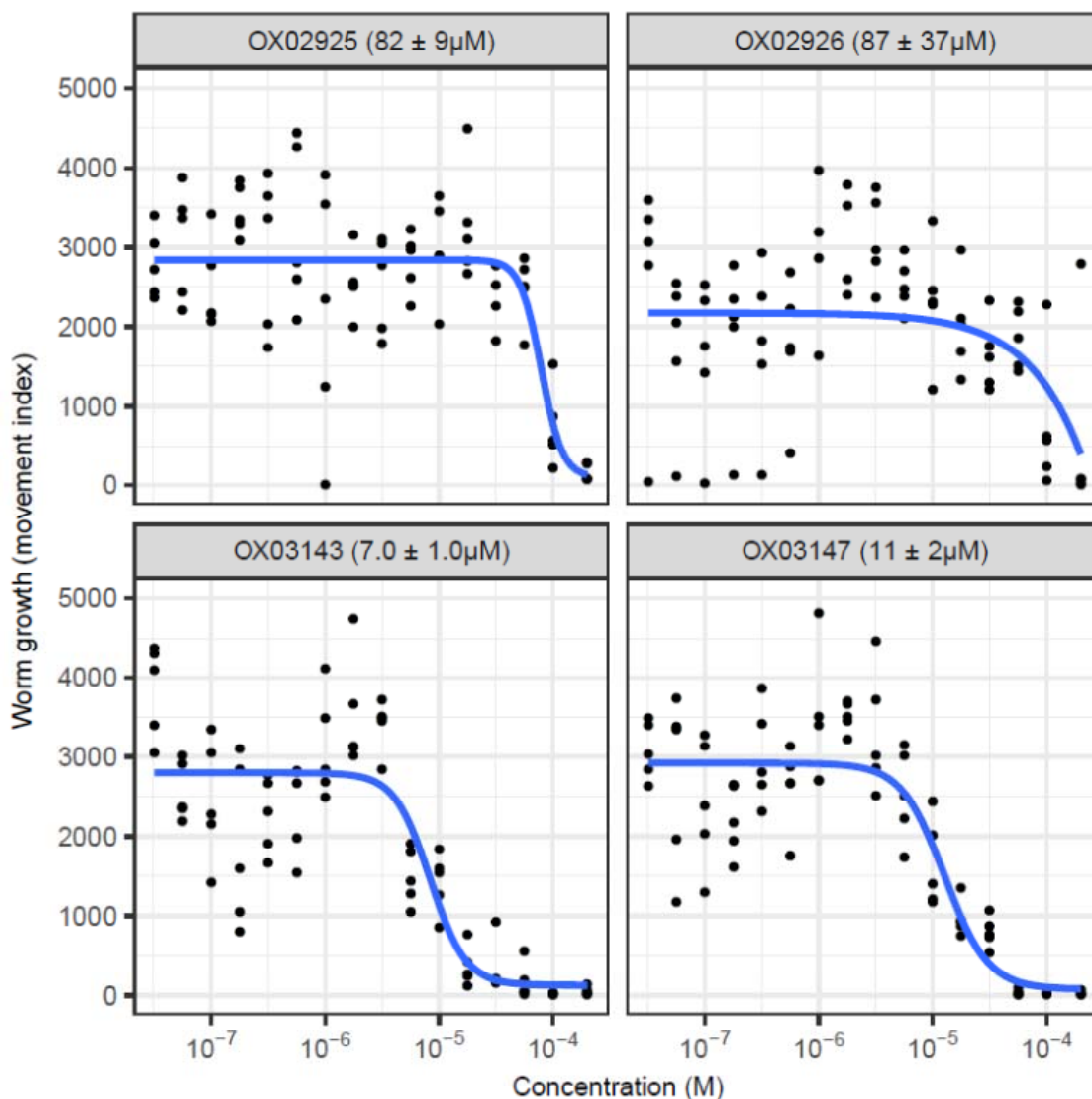
381 This class has ‘lead-like’ or ‘drug-like’ chemical properties [20], although it is important to note that  
382 in the contemporary medicinal chemistry literature this term is usually applied in the context of  
383 imparting oral bioavailability characteristics (Table 1). For agents targeting the gastrointestinal  
384 located *Trichuris*, minimal systemic exposure of the host is desirable and therefore it is critical to  
385 differentiate between the conventionally used terminology and parameters for ‘drug-like’ molecules,  
386 which affect solubility and permeability, compared to properties that would be relevant to agents  
387 targeting other body compartments. Recent literature has described this important caveat for non-  
388 peripheral CNS drugs [21], and indeed for anti-parasitic drug development [22]. Importantly, there is  
389 considerable scope for generating the large number of structural variants of the DATPs needed for the  
390 iterative improvement of compound properties during the downstream lead optimisation process.

391 Active diaminothienopyrimidines block *C. elegans* development

392 Although we are focused on developing an anthelmintic with improved efficacy over existing drugs  
393 against *Trichuris*, activity across the nematode phylum is valuable, particularly as efficacy against  
394 economically significant agricultural animal parasites would make further development more  
395 economically viable.

396 We therefore wanted to test the activity of the DATP chemotype against the clade V nematode  
397 *Caenorhabditis elegans*. Using a quantitative development assay to measure the growth of  
398 synchronised L1 stage worms, we tested varying concentrations of the compounds to determine the  
399 concentration-response effects. As shown in Fig 4, all four DATP compounds were active in this  
400 assay with EC<sub>50</sub> values from 7 – 87 μM.

401



402

403 **Fig 4. Concentration-response curves for resynthesized DATPs in the *C. elegans* growth assay.**

404 n=5 wells per concentration per compound, each replicate on a different 96-well plate. Blue line

405 indicates concentration-response curve fitted with the 4-factor log-logistic model using *drc* [16].

406 Figure in parenthesis indicates EC<sub>50</sub> estimate ± standard error from this model.

407 Interestingly, the DATPs display differing trends in activity between the *Trichuris* and *C. elegans*

408 assays. At this stage we do not know whether this reflects different potency at the target or different

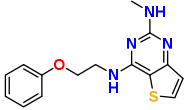
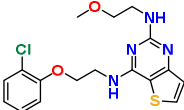
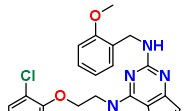
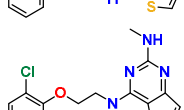
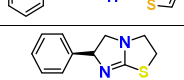
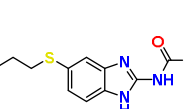
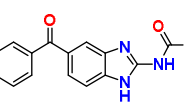
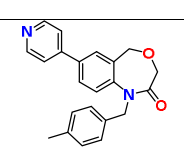
409 patterns of drug access between the species, but the findings highlight the importance of screening

410 against *Trichuris* in the search for novel anthelmintic agents targeting whipworm. The data from each

411 of these assays as well as structural descriptors and Lipinski rule assessment for the four DATP  
412 compounds and other anthelmintics are summarised in Table 1. The leading member of the  
413 dihydrobenzoxazepinone class OX02983 is shown in Table 1 for comparison. EC<sub>50</sub> values for the two  
414 series are currently in a similar range.

415

416 Table 1. Properties and activities of resynthesized diaminothienopyrimidines, and other anthelmintics

Compound	PubChem CID	Structure	EC <sub>50</sub> (μM)		RMM	cLogP	HBA	HBD	tPSA (Å <sup>2</sup> )	ROTb
			<i>T. muris</i> paralysis assay	<i>C. elegans</i> growth assay						
		('Drug-like' guidelines)			<500	<5	<10	<5	(<140)	(≤10)
<b>OX02925</b>	49790760		47 ± 3	82 ± 9	300	2.5	5	2	87	6
<b>OX02926</b>	49790669		27 ± 2	82 ± 37	379	3.0	6	2	97	9
<b>OX03143</b>	46948320		> 80	7 ± 1	440	4.5	6	2	97	9
<b>OX03147</b>	49778268		44 ± 2	11 ± 2	344	3.1	5	2	87	6
<b>Levamisole</b>	26879		8 ± 3 <sup>a</sup>	5 ± 1 <sup>a</sup>	204	1.7	2	0	41	1
<b>Albendazole</b>	2082		> 800 <sup>b</sup>	n.d.	249	2.3	6	2	76	5
<b>Mebendazole</b>	4030		>600 <sup>b</sup>	1.1 ± 0.2 <sup>a</sup>	295	2.7	6	2	84	4
<b>OX02983</b>	71447449		50 ± 13 <sup>c</sup>	n.d.	344	2.7	4	0	42	3



417 RMM: relative molecule mass. HBA: number of hydrogen bond acceptors. HBD: number of hydrogen bond donors. tPSA: topological polar surface area,  
418 calculated using DataWarrior [23]. ROTB: number of rotatable bonds. <sup>a</sup> Data from [15]. <sup>b</sup> Data from [24]. <sup>c</sup> Data from [12].

419 Assessment of the cytotoxicity of the diaminothienopyrimidine series

420 It was critical to ensure that this series of compounds showed minimal cytotoxicity towards  
421 mammalian cells, and showed selective activity against the parasite. For example, gut cytotoxicity  
422 may result in the compounds having too narrow a therapeutic window. Selected examples of the  
423 DATPs were assessed for cytotoxicity using the mouse gut epithelial cell line CMT-93 (Table 2).  
424 Although, the DATPs exhibited increased *in vitro* cytotoxicity in these assays compared to the  
425 previously reported DHB series [12], an encouraging overall profile was exhibited for these early stage  
426 molecules. Furthermore, the nematode cuticle often limits drug access which reduces target  
427 engagement by small drug-like molecules [25,26]. This means that compound optimisation to improve  
428 uptake through the cuticle may be a fruitful route to improved anti-nematode selectivity, as well as  
429 improving the cytotoxicity profile.

430 It is interesting to note that the activity against *Trichuris* did not correlate with cytotoxicity, with the  
431 most cytotoxic compound (**OX03143**) showing the lowest activity in the *T. muris* adult paralysis  
432 assay, with an  $EC_{50} > 80\mu\text{M}$ . This suggests that either anti-*Trichuris* activity is distinct from cytotoxic  
433 action, or that differential drug access can be exploited to achieve differential host-parasite activity.  
434 Either possibility is encouraging and suggests that continued exploration and iterative improvement of  
435 the DATP structure might be anticipated to deliver a more potent anthelmintic with acceptable host  
436 toxicity.

437

Compound	WST-8 $EC_{50}$ ( $\mu\text{M}$ )	Neutral red $EC_{50}$ ( $\mu\text{M}$ )	Adult <i>Trichuris</i> paralysis assay $EC_{50}$ ( $\mu\text{M}$ )	438
<b>OX02925</b>	75 (48-124)	29 (19-43)	47	
<b>OX02926</b>	43 (28-67)	21 (15-31)	27	439
<b>OX03143</b>	15 (9-26)	5 (3-7)	>80	
<b>OX03147</b>	37 (24-57)	21 (14-30)	44	440

441 **Table 2. Summary of the cytotoxicity in a mouse epithelial cell line of the DATP series.** Mouse  
442 CMT-93 rectal epithelial cells were used for this assay. Maximum tested concentration was 100  $\mu\text{M}$ .

443  $n=8$ , error range (in parentheses) shows 95% confidence interval. EC<sub>50</sub> values in the adult *Trichuris*  
444 paralysis assay are shown for comparison.

#### 445 Activity of diaminothienopyrimidines against the infective egg stages of *T. muris*

446 Developing novel anthelmintics to disrupt the *T. trichiura* life cycle at the egg stage represents an  
447 exciting and complementary strategy to an oral therapy and is particularly attractive as *T. trichiura*  
448 eggs are highly resistant to extreme temperature changes and ultraviolet radiation, thereby remaining  
449 viable in the environment for many years [27]. We assessed whether the DATP derivatives were  
450 capable of affecting either infection establishment or embryonation of eggs. We first explored whether  
451 the compounds could alter the establishment of infection by soaking embryonated *T. muris* eggs in the  
452 test compounds for 14 days, washing the eggs and then determining infectivity both *in vitro* and *in*  
453 *vivo* (Fig 5a).

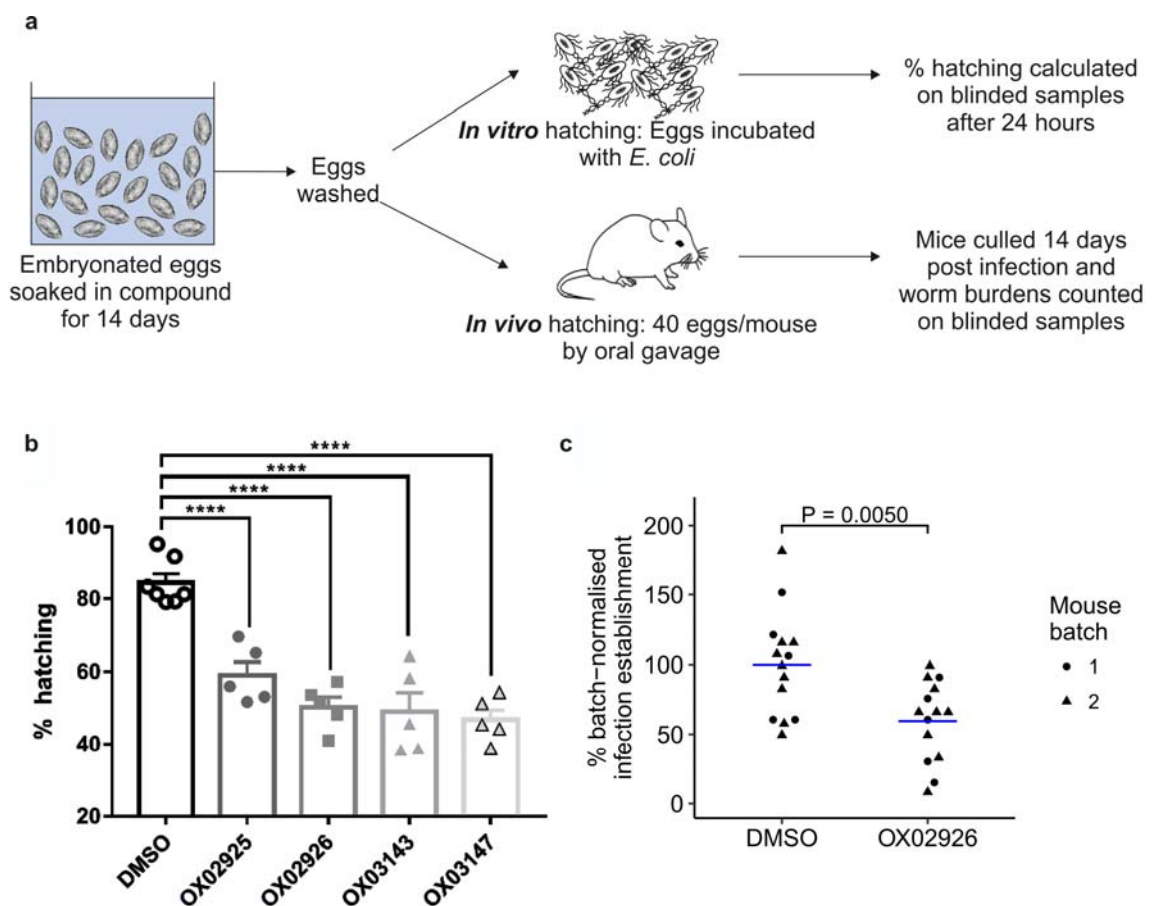
454 To determine effects on *in vitro* hatching, a protocol modified from that previously described [8] was  
455 established whereby eggs were induced to hatch when incubated in a culture of *Escherichia coli* at 37  
456 °C. The results are summarised in Fig 5b. Strikingly all DATPs were capable of significantly reducing  
457 *in vitro* hatching compared to the DMSO control.

#### 458 Diaminothienopyrimidines reduce the ability of *T. muris* eggs to infect mice

459 To extend this finding, we selected **OX02926** to test in an *in vivo* hatching and infection establishment  
460 assay, as this compound showed both a significant decrease in *in vitro* hatching and a small standard  
461 deviation between samples. The eggs were soaked as for the *in vitro* experiment and SCID mice were  
462 infected with 40 treated eggs (**OX02926** or DMSO) by oral gavage. Egg infectivity was quantified at  
463 day 15 post infection by culling the mice and counting the number of established L2 larvae in the gut.  
464 All L2 larvae counted had a normal morphology as viewed under a dissecting microscope.

465 This experiment was carried out in two batches and the raw data are shown in the S2 Figure. Because  
466 variation in control worm establishment is commonplace in *Trichuris* infections due to natural  
467 variation in egg infectivity from a standardised egg number, we took the approach of normalising data

468 for each batch relative to the mean of the DMSO-only control group for that batch. This allowed us to  
469 determine the effects of **OX02926** treatment (a full statistical description is given in the Methods  
470 section). The results are shown in Fig 5c. We used a two-way ANOVA (% batch normalised infection  
471 establishment ~ treatment \* batch) to analyse the data. There was a significant effect of treatment  
472 [F(1,24) = 9.569, P = 0.00497] but no effect of batch [F(1,24) = 0.083, P = 0.77618] or interaction  
473 [F(1,24) = 0.083 0.77618]. We therefore conducted a post-hoc Tukey HSD test which showed that  
474 infection establishment in the **OX02926**-treated group was significantly different from the DMSO-  
475 treated control group (P = 0.0050). Treatment of eggs with **OX02926** was able to significantly reduce  
476 the burden of worms *in vivo* by an estimated 40%. This is likely to reflect reduced infectivity of  
477 DATP-treated eggs.



478

479 **Fig 5. Reduced worm burden in mice given *T. muris* eggs that had been treated with**  
480 **diaminohienopyrimidines. (a)** Embryonated eggs were soaked in compound for 14 days, washed in  
481 water and then used in either *in vitro* or *in vivo* hatching assays. **(b)** Treatment with DATPs reduced  
482 the ability of embryonated eggs to hatch in *E. coli* bacterial suspension after 24 hours. A one-way  
483 ANOVA showed a significant difference between treatment groups ( $F(5,26)=25.95$   $p<0.0001$ ) with a  
484 post-hoc Dunnett's compared to DMSO control (\*\*\*\*=  $p<0.0001$ )  $n=7$  (DMSO),  $n=5$  (DATP  
485 compounds) **(c)** SCID mice were infected with 40 eggs and worm burden assessed at day 15 post  
486 infection. The experiment was carried out in two batches, with  $n=5$  and  $n=9$  mice respectively in each  
487 of the control and treatment groups. Data were normalised for each batch relative to the mean of the  
488 DMSO-only control group for that batch. Blue line indicates mean for each treatment group. A two-  
489 way ANOVA showed a significant effect of treatment [ $F(1,24) = 9.569$ ,  $P = 0.00497$ ] but no effect of  
490 batch [ $F(1,24) = 0.083$ ,  $P = 0.77618$ ] or interaction [ $F(1,24) = 0.083$   $0.77618$ ]. A post-hoc Tukey HSD  
491 test showed that the **OX02926**-treated group was significantly different from the DMSO control group  
492 ( $P = 0.0050$ ).

493

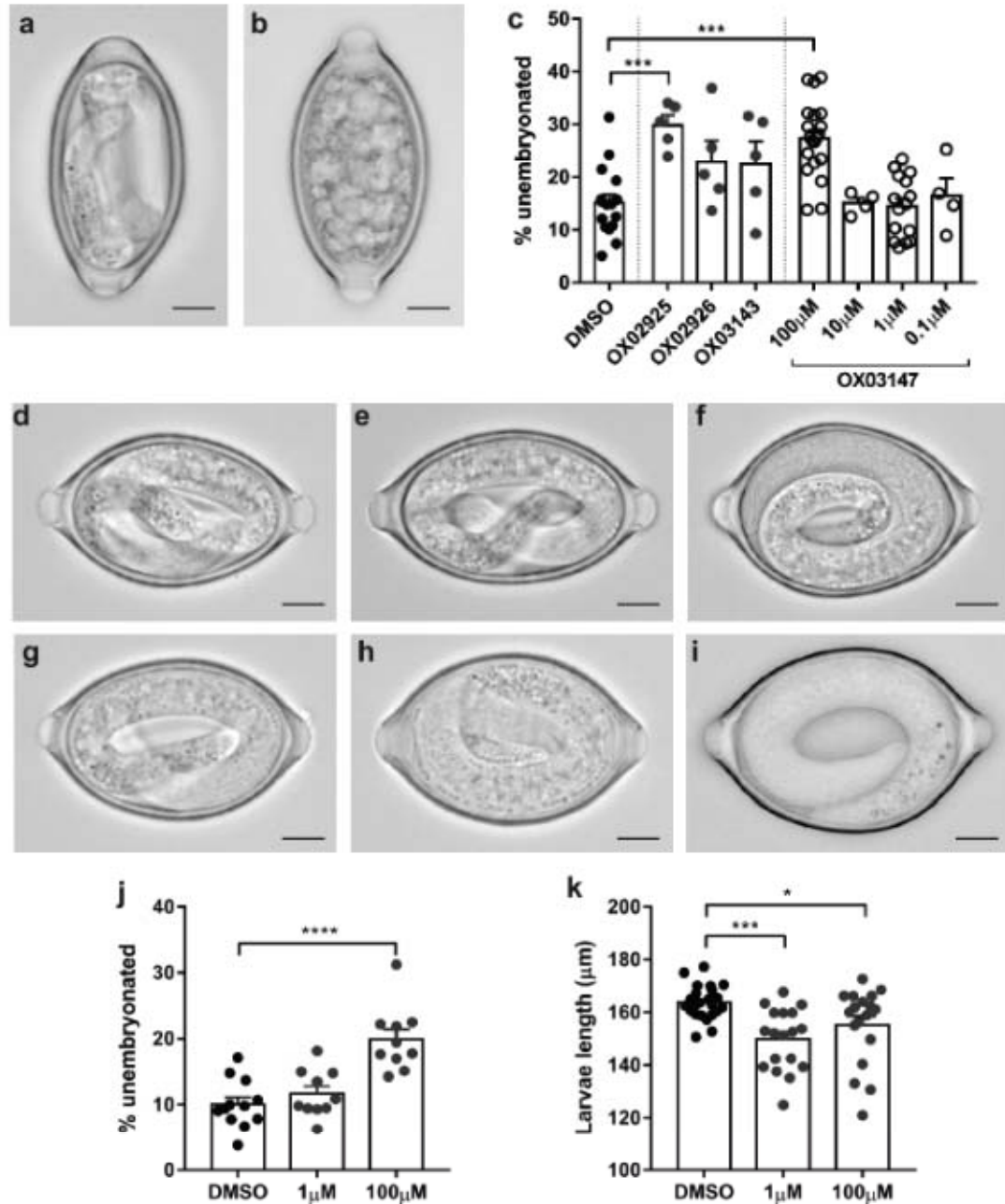
494 Activity of diaminohienopyrimidines against the embryonation of *T. muris* eggs

495 The ability of the DATPs to alter the embryonation of *T. muris* eggs was investigated by soaking  
496 unembryonated *T. muris* eggs collected overnight from live adult *T. muris* in the test compounds at 26  
497 °C for the duration of the embryonation process (56-60 days). During embryonation the first larval  
498 stage of the parasite develops within the egg shell (Fig 6a) from a ball of cells (Fig 6b). Treatment  
499 with the DATPs **OX02925** and **OX03147** resulted in a significant increase in the percentage of  
500 unembryonated eggs present compared to the DMSO control (Fig 6c). Importantly, although the other  
501 DATPs did not alter the percentage of eggs unable to undergo the embryonation process, the larvae  
502 that developed were atypical (Fig 6d-i). These atypical larvae were morphologically altered with the  
503 granules present within the larvae appearing less distinct.

504 As **OX03147** had the clearest phenotype with a significant increase in the number of unembryonated  
505 eggs, a concentration response study was performed to determine if an effect could be seen at lower  
506 treatment doses. Additionally, we repeated the experiment at room temperature to allow for more  
507 physiological conditions rather than the constant 26 °C utilised in the initial study to standardise  
508 conditions across experiments. Although the increased number of unembryonated eggs was only  
509 detected at the highest drug dose tested (100 µM) at both 26 °C and room temperature (Fig 6c, j)  
510 striking effects on egg morphology was detectable at concentrations as low as 1 µM with significant  
511 larval stunting observed (Fig 6k).

512 To determine if an effect on embryonation could be observed following a shortened drug exposure we  
513 soaked unembryonated eggs in 100 µM OX03147 at 26 °C for weeks 0-2, 0-3, 2-4 or 4-6 of  
514 embryonation. Although there was no increase in the proportion of unembryonated eggs observed in  
515 any treatment group (S3 Figure f) there were clear morphological alterations in the L1 larvae within  
516 the egg following exposure to OX03147 during weeks 0-3, 2-4 or 4-6 of the embryonation process (S3  
517 Figure a-e). The most striking observation was the clear larval stunting observed following drug  
518 soaking from weeks 0-3 (S3 Figure c, g). A one-way ANOVA test showed a significant effect of  
519 treatment on larval length,  $F(4, 21) = 3.984, P=0.0147$ . A post-hoc Dunnett's test showed a significant  
520 difference in the Weeks 0-3 treatment group compared to the DMSO-only control group ( $P = 0.0076$ ).  
521 This appeared to phenocopy the effect OX03147 had when treated for the duration of the  
522 embryonation process at 1 µM (Fig 6k). Additionally, in the 2-4 week and 4-6 week groups, although  
523 larval length was not affected, there was evidence of structural alterations in the L1 larvae with a less  
524 distinct structure present and altered granulation within the larvae (S3 Figure d, e).

525 To the known range of applications of DATPs in medicinal chemistry we can now add anthelmintic  
526 activity. This study suggests they have significant potential for further development into dual-acting  
527 therapeutic agents for both the reduction of *Trichuris* egg infectivity, and embryonation in the  
528 environment. Thus, their actions on both the embryonated and unembryonated egg stages may enable  
529 a break in the parasite lifecycle.



530

531 **Fig 6. Unembryonated *T. muris* eggs treated with diaminothienopyrimidines have altered**  
532 **embryonation.** Unembryonated eggs were soaked in 100 μM compound (unless specified otherwise)  
533 at 26 °C (unless specified otherwise) for the duration of the embryonation process (56-60 days) and  
534 then embryonation determined and eggs imaged using an Olympus BX63 microscope. Scale bar  
535 indicates 10 μm. (a) Typical embryonated egg and (b) unembryonated egg. (c) treatment with DATPs  
536 increased the incidence of unembryonated eggs. Representative pictures of (d) DMSO, (e) OX02925,

537 (f) **OX02926**, (g) **OX03143** and (h) **OX03147** 100  $\mu$ M and (i) **OX03147** 1  $\mu$ M soaked *T. muris* eggs.  
538 (j) Unembyronated eggs soaked in **OX03147** at room temperature for 56 days and embryonation  
539 determined. (k) Unembyronated eggs soaked in **OX03147** at 26 °C for 56 days and larval length  
540 calculated using ImageJ.

541



## 542 **Discussion**

543 Gastrointestinal nematode parasites remain a significant human health burden. Current anthelmintics  
544 lack efficacy and achieve low cure rates, threatening the targets set by the World Health Organisation  
545 for control of soil-transmitted helminths [2,28]. In particular, existing drugs have notably low efficacy  
546 against *T. trichiura*, the human whipworm. *T. trichiura* may be especially difficult to target as it  
547 inhabits the large intestine and is in part intracellular [29]. The metabolically active anterior of the  
548 worm, the stichosome, is buried in the host epithelial cells lining the gut, affording some protection  
549 from orally delivered anthelmintics.

### 550 Diaminothienopyrimidines (DATPs), a new anthelmintic chemotype

551 We recently reported a small molecule screen for new anthelmintics targeting the gastro-intestinal (GI)  
552 nematode parasite *Trichuris muris* that identified the dihydrobenzoxazepinone (DHB) chemotype. The  
553 DHBs had not previously been ascribed anthelmintic activity [10]. Here, we describe a second class of  
554 novel anthelmintic, the diaminothienopyrimidines (DATPs). The potential for this early stage series is  
555 significant; their chemical synthesis is facile and lends itself to iterative optimisation, which will  
556 facilitate structural modifications aiming, for example, to increase local epithelial penetrance and  
557 hence improve efficacy during future development. Furthermore, their straightforward production  
558 imparts a favourable cost benefit aspect to the series.

### 559 Other thienopyrimidines – their applications and targets

560 Thienopyrimidines have received much interest in medicinal chemistry as they are bioisosteres for  
561 purines, such as the nucleic acid components adenine and guanine. They are also related to  
562 quinazolines, an important class of kinase inhibitors, including gefitinib and erlotinib, which act by  
563 recognizing the ATP-binding site of the enzyme [30]. Thieno[2,3-*d*]pyrimidines are a particularly  
564 important scaffold, with many reported examples of protein kinase inhibitors, as well as inhibitors of  
565 dihydrofolate reductase, kainate receptor agonists, and  $\alpha_1$ -adrenoreceptor antagonists [31].

566 The thieno[3,2-*d*]pyrimidine scaffold found in the compounds reported in this study, has also been  
567 investigated. A series of 2-aryl 4-morpholino derivatives have been identified as phosphatidylinositol-  
568 3-kinase inhibitors [32], leading to the discovery of the PI3K inhibitor GDC-0941 (pictilisib) [33] and  
569 the dual PI3K/mTOR inhibitor DGC-0980 (apitolisib) [34]. The structures of these compounds are  
570 shown in the S4 Figure, in comparison with the 2,4-diaminothieno[3,2-*d*]pyrimidine OX02926.  
571 Pictilisib and apitolisib are under development as anti-cancer agents, have been tolerated in Phase I  
572 trials for solid tumors, and Phase II trials have commenced [35,36].

573 A series of 2,4-diaminothieno[3,2-*d*]pyrimidines have been described as orally active antimalarial  
574 agents [37], with activity in the low nanomolar range against *Plasmodium falciparum*. The structures  
575 of these compounds are shown in the S4 Figure in comparison with OX02926. This anti-malarial  
576 series was later improved by systematic modification giving improved antimalarial activity, but  
577 unfortunately continued hERG inhibition [38]. Whilst our DATP compounds have the same core  
578 scaffold as the anti-malarial series, they have different substituents, and in particular lack the 6-aryl  
579 substituent that is critical for anti-malarial activity and found in all compounds tested for hERG  
580 activity. However, the authors were able to demonstrate that hERG activity could be removed through  
581 modification of the C1 substituent, suggesting that this potential liability is not intrinsic to the 2,4-  
582 diaminothieno[3,2-*d*]pyrimidine core. We have not yet performed hERG assessment of our  
583 compounds, but this will form an important part of the future development of this series.

584 A series of 2,4-diaminothieno[3,2-*d*]pyrimidines has also recently been reported as active against the  
585 endosymbiotic bacterium *Wolbachia*, with potential use against filarial nematodes [39]. In neither the  
586 anti-malarial or anti-*Wolbachia* case is the molecular target of the compounds known.

587 DATPs, their potential and route to a new anti-whipworm oral therapy

588 The major goal of our research is to develop a new oral therapy for trichuriasis, which could be widely  
589 used in mass drug administration programs leading to the eradication of human whipworm. Such an  
590 agent should have a substantially higher single-dose cure rate than the current drugs used in mass drug

591 administration, albendazole and mebendazole. Drug development is long process, and recent work has  
592 defined a set of criteria, tailored to neglected infectious diseases, for progression in the hit to lead and  
593 lead optimisation stages [40,41]. Our DATP series members are early-stage compounds in the  
594 development process. The compounds meet almost all of the criteria for hit selection in neglected  
595 diseases, including confirmed activity with resynthesized material, dose-dependent *in vitro* activity, a  
596 tractable chemotype that passes drug-likeness filters such as the Lipinski rule of five, and an  
597 established synthetic route of only two steps [40]. The most pressing weakness of the series is the  
598 small selectivity window for their activity against the parasite compared to cytotoxicity in a  
599 mammalian cell line. Improving this property for these early stage compounds must be a priority for  
600 future development. The DATP compounds also meet some of the milestones in the hit to lead  
601 process, particular in terms of drug-likeness and the exploitability of the structure, giving the ability to  
602 generate variants and establish the structure-activity relationship and hence improve potency and  
603 selectivity [41]. The *in vitro* activity of OX02926 in the adult whipworm motility assay ( $EC_{50} = 27\mu M$ ,  
604 equivalent to  $10.2\mu g/ml$ ) also reaches the activity threshold for lead compounds that has been  
605 determined for drug development against the microfilarial nematode *Brugia malayi* [41]. In summary  
606 the DATP series are promising early-stage compounds with a number of lead-like features.  
607 Improvement of potency, together with an understanding of parasite/host selectivity and  
608 pharmacokinetic properties will be the focus of the next steps of development.

#### 609 Activity against the egg stage of *T. muris*

610 In addition to activity against the adult stage of whipworm, the DATPs were also able to significantly  
611 reduce egg hatching, both *in vitro* and *in vivo*. These data are in keeping with members of the DHB  
612 series, which also were able to inhibit parasite egg hatching. However, unlike the DHB series, we  
613 identified members of the DATPs that also significantly reduced the percentage of eggs embryonating  
614 *ex vivo*, with other members of the DATP series appearing to disrupt the embryonation process,  
615 resulting in defects in embryonic elongation and abnormal egg shape. *Trichuris* egg embryonation  
616 occurs gradually and the mechanism by which it occurs is currently a poorly understood process. A  
617 detailed characterisation of the morphological changes which occur with the *Trichuris suis* egg during

618 embryonation has been described and other *Trichuris* species appear to undergo the same process.  
619 Once the unembryonated, unsegmented eggs are deposited, the two clear, nuclei-like areas move  
620 together and fuse. Cellular division then begins, initially occurring asymmetrically with two  
621 blastomeres of unequal size. The larger blastomere then divides again and then subsequently each  
622 blastomere divides in two until a blastula formed of many small blastomeres develops. The initial  
623 larval differentiation then occurs with the appearance of a motile cylindrical embryo, which gradually  
624 turns into an infective larva with its characteristic oral spear. The fully developed larva is no longer  
625 motile and is thought to be an L1 larva as no moult is observed within the egg [42]. The embryonation  
626 process is temperature sensitive. The effect of temperature on egg embryonation has been  
627 characterised in detail in recent years for *T. suis* eggs with the embryonation process accelerated at 30-  
628 32 °C compared to 18 °C, with degeneration of the eggs rather than embryonation observed at higher  
629 temperatures (40 °C). At low temperatures (5-10 °C) no embryonation occurs, however once these  
630 eggs are then transferred to optimal embryonation temperatures normal embryonation proceeds [43].  
631 Similar temperature sensitivity has been described for other *Trichuris* species including *Trichuris*  
632 *trichiura* with different species embryonating with different kinetics [44,45]. More research is required  
633 to understand the mechanisms behind this embryonation process, which may then allow an even more  
634 targeted approach to breaking the life cycle.

#### 635 Potential and feasibility of an environmental treatment

636 Humans become infected with *Trichuris* via a faecal oral route. Adult parasites in the intestine shed  
637 unembryonated eggs, which pass out with the faeces and embryonate in the external environment over  
638 a period of five weeks. Eggs can remain viable in the environment for many months [46]. Parasite  
639 eggs are only infective if fully embryonated upon ingestion. Thus, the ability of the DATPs to disrupt  
640 both the infectivity of embryonated eggs and the embryonation process itself suggests a potential  
641 environmental control to decrease *Trichuris* infection rates in the field without the need to develop and  
642 administer a new oral anthelmintic to the infected population.

643 In particular, it has been noted that the environmental pool of infectious eggs makes those individuals  
644 successfully treated, typically once or twice per year, in mass drug administration programs at risk of  
645 reinfection [47]. It has therefore been proposed that improvements in sanitation are required in  
646 addition to anthelmintic MDA. We suggest that an environmentally-acting, egg-targeting agent,  
647 potentially developed from our DATP series compounds, could play a complementary role to help  
648 break transmission in parallel with MDA and sanitation improvements.

649 Clearly it is not possible to widely treat large areas of endemic regions with such an environmental  
650 control. Instead, we envisage the targeted use of DATPs in the environment at sites of high parasite  
651 egg density; these might include for example focusing treatment around pit latrines, as it is known that  
652 pit latrines may be a focal point of infection with a high concentration of eggs of soil-transmitted  
653 helminths [48]. In a study in Ethiopia, *Trichuris trichiura* prevalence was higher in communities with  
654 greater latrine usage (compared to field or yard defecation), suggesting that basic pit latrines may in  
655 some circumstances be ineffective at reducing infection [49]. However improved sanitation facilities  
656 generally, including pit latrines, ventilated improved pit latrines, and flush toilets, do reduce STH  
657 infection rates [47,50].

658 Such an egg-targeted agent should have a limited negative effect on the environment, have a suitable  
659 formulation for practical delivery, and be able to block egg viability at low concentration in the  
660 environment. The DATP series, which damage egg development and infectivity when applied at fairly  
661 high concentrations (1 to 100 $\mu$ M) for quite long periods of time (from 2 to 3 weeks to 60 days) show  
662 potential for developing such an agent. However these properties need to be improved during future  
663 development, while achieving an appropriate safety and environmental profile.

664

## 665 Conclusions

666 In summary we report the discovery of a new class of anthelmintic, the DATPs, which possesses  
667 activity directed against adult stage *T. muris* parasites and the egg stage. Importantly, as a chemical

668 series the DATPS are notable, since they are relatively facile to produce synthetically thereby  
669 presenting considerable scope for structural modifications to improve efficacy and deliver an  
670 optimised agent.

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674

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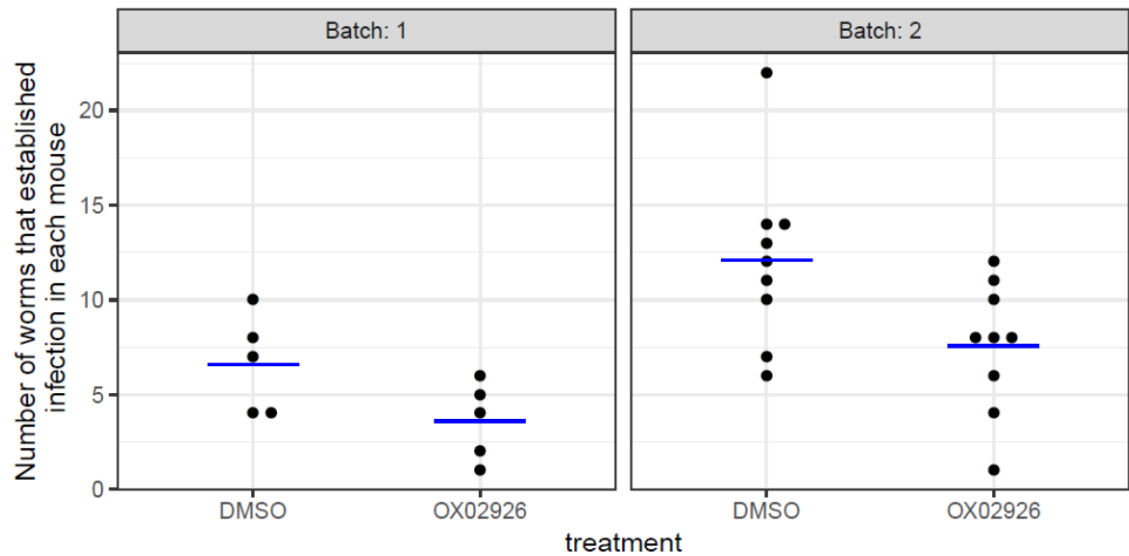
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826 **Supporting information captions**

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828 **S1 File. <sup>1</sup>H NMR, <sup>13</sup>C NMR and HRMS Spectra supporting the synthesis of the DATP**

829 **compounds**



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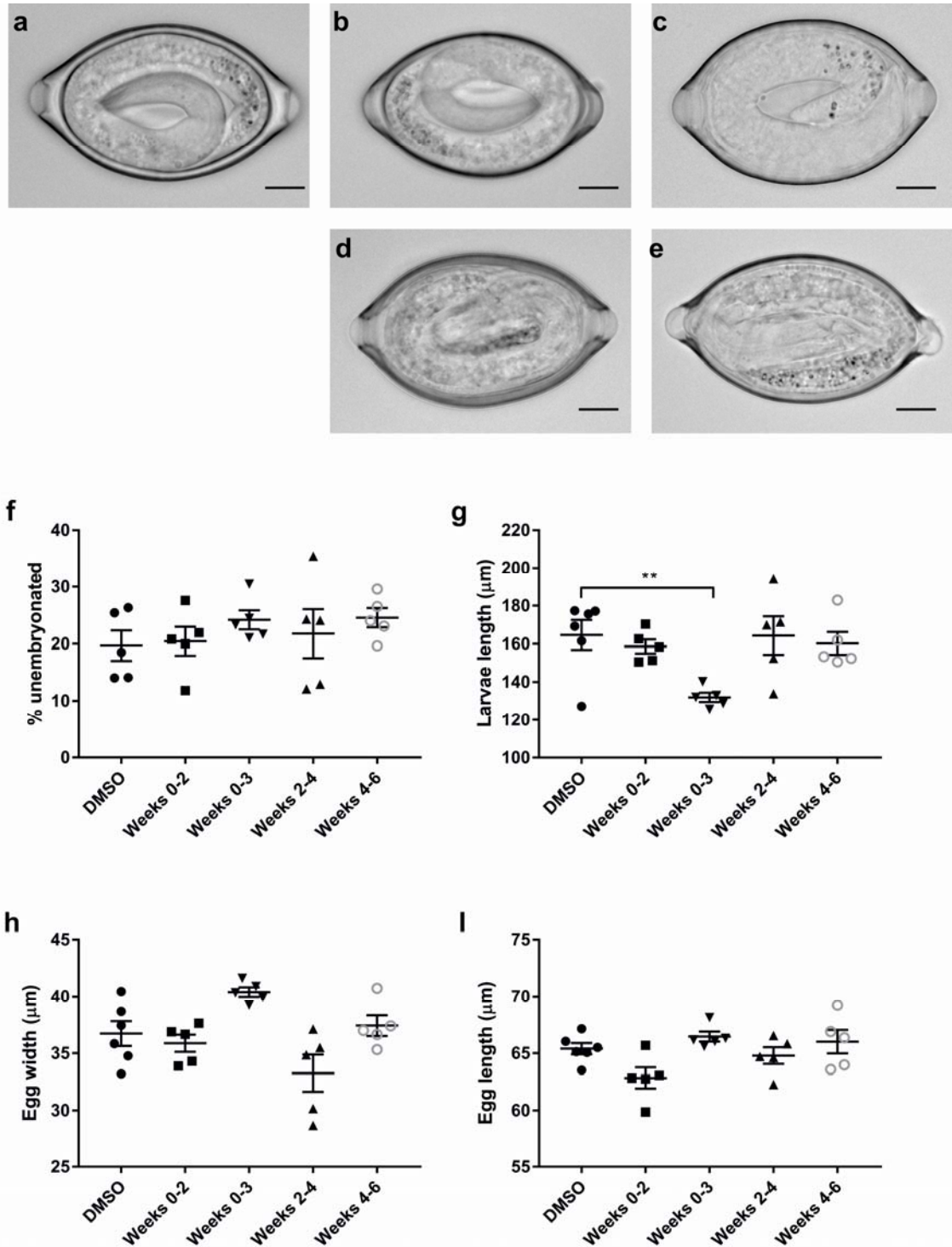
831 **S2 Figure. Raw data separated by batch for the in vivo hatching experiment.** Each point indicates

832 one mouse that has been infected with *T. muris* eggs that had been treated with deionised water plus

833 1% v/v DMSO (control) or deionised water plus 1% v/v DMSO and final concentration 100 $\mu$ M

834 OX02926 for 14 days. Blue line indicates mean for each treatment group.

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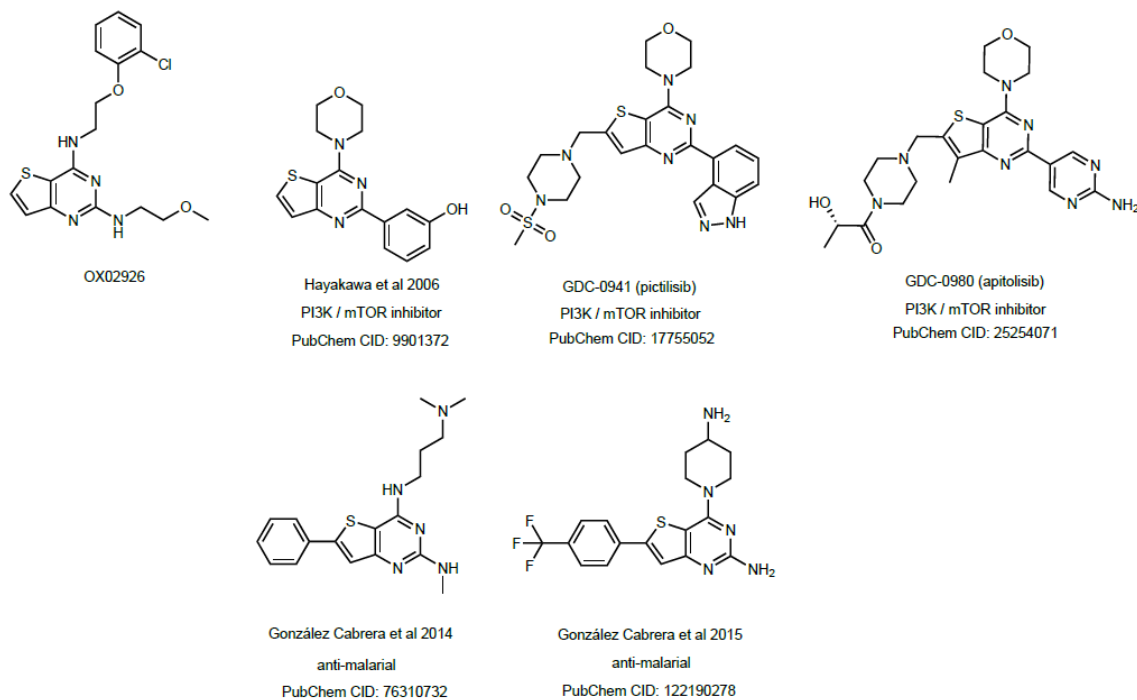


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837 **S3 Figure. Unembryonated *T. muris* eggs treated with OX03147 for part of the embryonation**  
838 **process have altered morphology.** Unembryonated eggs were soaked in 100  $\mu\text{M}$  OX03147 at 26°C  
839 for the duration specified and then embryonation determined and eggs imaged using an Olympus

840 BX63 microscope. Scale bar indicates 10  $\mu\text{m}$ . Representative pictures of (a) DMSO, (b) OX03147  
841 weeks 0-2, (c) OX03147 weeks 0-3, (d) OX03147 weeks 2-4, (e) OX03147 weeks 4-6. Following 56  
842 days embryonation was determined (f) and larvae length (g), egg width (h) and egg length (i)  
843 calculated using ImageJ. \*\* Indicates  $P < 0.01$ , one way ANOVA with post-hoc Dunnett's test  
844 compared to DMSO control.

845



846

847 **S4 Figure. Structures of selected thieno[3,2-d]pyrimidine compounds in development compared**  
848 **to OX02926**