High-throughput screening of more than 30,000 compounds for anthelmintics against gastrointestinal nematode parasites

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Abstract:

Gastrointestinal nematodes (GINs) are amongst the most common parasites of humans, livestock, and companion animals. GIN parasites infect 1-2 billion people worldwide, significantly impacting the growth, health, and vitality of hundreds of millions of children, pregnant women, and adult workers in developing countries, thereby perpetuating poverty. The cost and losses associated with GIN infections in human and veterinary health are estimated to be hundreds of billions of dollars worldwide. Two benzimidazoles with suboptimal efficacy are currently used to treat GINs in humans as part of mass drug administrations, with many additional instances of lower-than-normal efficacy and possible resistance. Many livestock and companion animal GINs are resistant to multiple classes of anthelmintic drugs used today. Thus, new anthelmintics are urgently needed. However, screening methods for new anthelmintics using human GINs typically have a low throughput. Here, using our novel screening pipeline that starts with human hookworm parasites, we screened 38,293 conditions in duplicate representing 30,238 unique small molecules from a wide range of compound libraries, including generic diversity, repurposing, natural derivatives, mechanism of action, and multiple target-focused libraries (*e.g.*, targeting kinases, GPCRs, and neuronal proteins). We identified 55 compounds with broad-spectrum activity against adult stages of two evolutionary divergent GINs, hookworms (*Ancylostoma ceylanicum*) and whipworms (*Trichuris muris*). Based on known databases, the targets of these 55 compounds were predicted in nematode parasites. One novel scaffold from the diversity set library, F0317-0202, showed good activity (≥~11% Relative motility) against both GINs. To better understand this novel scaffold's structure-activity relationships (SAR), we screened 28 analogs and created SAR models highlighting chemical and functional groups required for broad-spectrum activity. These studies validate our new and efficient screening pipeline at the level of tens of thousands of compounds and provide an

important set of new GIN-active compounds for developing novel and broadly-active anthelmintics.

Keywords:

Gastrointestinal nematode parasites, soil-transmitted helminths, anthelmintic discovery, drug screening, hookworms, whipworms

85 **Introduction:**

86 Human gastrointestinal nematodes (GINs, also known as soil-transmitted helminths) include the
87 roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura*, and the hookworms
88 *Necator americanus*, *Ancylostoma duodenale*, and *Ancylostoma ceylanicum*; they are amongst
89 the most prevalent infections of the so-called Neglected Tropical Diseases (NTDs)[1], affecting
90 people in the world's poorest communities[2-4]. GINs are successful parasites of vertebrates,
91 adapted to parasitize humans, livestock, and companion animals[5]. Morbidities associated with
92 GIN infections in humans include anemia, malnutrition, growth stunting, cognitive impairment,
93 pregnancy complications, and lowered immunity against other infectious diseases[2, 4, 6]. The
94 negative impacts of GINs are hard to measure, but they are minimally responsible for > 2-3
95 million disability-adjusted life years (DALY)[3, 6, 7], with hookworms accounting for almost one-
96 half of those DALYs[3, 6]. Children who received hookworm treatment had increased school
97 attendance and adult income[4, 8]. GINs also have enormous impacts on the health and productivity
98 of farm animals[9].

99

100 The World Health Organization (WHO) has recommended annual or semi-annual Mass Drug
101 Administration (MDA) targeting preschool and school-age children to reduce worm burden and
102 associated morbidity[10]. Four anthelmintics (levamisole, pyrantel pamoate, albendazole, and
103 mebendazole), initially developed for livestock, are currently on the WHO list to control human
104 GINs, with the two benzimidazoles, albendazole and mebendazole, most widely used because of
105 their better efficacies and ease of dosing[11, 12]. In companion and livestock animals, GINs are
106 controlled via frequent and routine treatment using a repertoire of anthelmintics, which has led to
107 widespread and sometimes multi-drug anthelmintic resistance[13-16]. Benzimidazole resistance is

108 well-established in veterinary parasites[17-20]. Single nucleotide polymorphisms (SNPs) in the beta-
109 tubulin gene linked to the benzimidazole resistance in veterinary parasites have been found in
110 human GINs[21-24], indicating that with high drug pressure, there is likely to be an emergence for
111 benzimidazole resistance in human GINs. In Haiti and Kenya, the frequency of benzimidazole-
112 resistance codon 200 in *T. trichiura* was significantly increased following local MDA
113 programs[22]. There are also multiple reports of lowered efficacy of benzimidazoles against human
114 GINs and warnings against drug resistance[25-27]. Notwithstanding these challenges, the WHO has
115 set an ambitious goal to eliminate human GINs by 2030[10]. Therefore, potent and broadly active
116 anthelmintics with new modes of action are urgently needed for long-term control and
117 elimination. One promising new drug is emodepside, which was discovered >30 years ago [28].
118 However, it has not yet been approved for human use, and, as with all single anthelmintics,
119 resistance is a concern[29, 30]. Given the lead time for new drugs to enter the clinic, it is urgent that
120 new lead anthelmintics be put into the pipeline.
121
122 Because understanding of these parasites' biology is still limited, whole organism phenotypic
123 screening remains a preferred approach for anthelmintic drug discovery[31, 32]. Phenotypic
124 screening using GIN parasites for anthelmintic has included screening against *ex vivo* adult
125 parasites, egg hatch assay, larval stages, and infective third-stage larvae[32]. However, regarding
126 GINs that infect humans, the throughput of these screens has been limited[32-35]. The free-living
127 nematode *Caenorhabditis elegans* has been used as a surrogate model for GINs that infect
128 humans[32, 36-39]. However, we found limitations to using *C. elegans* as a model for anthelmintic
129 screening targeting human GINs, most notably a significant false negative hit rate[31]. Thus, *C.*
130 *elegans* is likely to miss some of the most promising compounds. We devised an alternative

screening model based on the larval stages of human hookworm parasites, which we found predicted better anthelmintic activity against GIN adult parasites than *C. elegans* [31]. In our pipeline, this is followed by screening against adult stages of evolutionarily divergent hookworm and whipworm parasites[31]. In a pilot screen, 1,280 compounds were screened as proof of concept[31].

Here, we scale up this anthelmintic discovery pipeline and screen 38,293 conditions (76,586 in duplicate) representing 30,238 unique compounds. We identified 55 small molecules with *in vitro* efficacy against adult hookworms and whipworms. We focused on one novel scaffold and performed Structure-Activity Relationship (SAR) studies. Based on analogies from other systems, we propose essential GIN targets for further studies.

Figure 1: Novel anthelmintic discovery pipeline for human GINs. Our pipeline is centered around the zoonotic hookworm, *A. ceylanicum,* as a primary (larval) and secondary (adult) screening model, followed by tertiary screening against the murine whipworm *Trichuris muris* (adult) to identify broadly active compounds. Hits are subjected to deep data mining, and analogs of potential candidates are tested to generate the Structure-Activity Relationships. This report covers the results from the first five stages of the screening pipeline (in blue).

Results:

We previously described and validated a pilot scale pipeline to discover anthelmintics that broadly target human GINs (Figure 1)[31]. We scaled up our primary screen with modifications to include 30,000+ compounds in 13 chemical libraries (Figure 2). Starting with the first larval (L1) staged *A. ceylanicum* hookworms, we screened these libraries, encompassing many structural features, including diversity scaffolds, natural product derivatives, repurposing drugs, known modes of action, and target-based libraries (Table 1). In total, we screened 30,238 unique

151 compounds in duplicate. Some compounds were screened at multiple doses due to plate format
152 availability, making the total number of individual conditions screened 38,293 (76,586 when
153 considered in duplicate). All libraries were initially screened in duplicate at 10 µM. As per our
154 pipeline, primary screen hits (in both duplicate wells) were screened at 30 µM against adult *A.*
155 *ceylanicum* hookworms. Adult hookworm hits were then screened at 30 µM against adult *T.*
156 *muris* whipworms. A breakdown of hits "library by library" is given below and summarized in
157 Table 1.
158

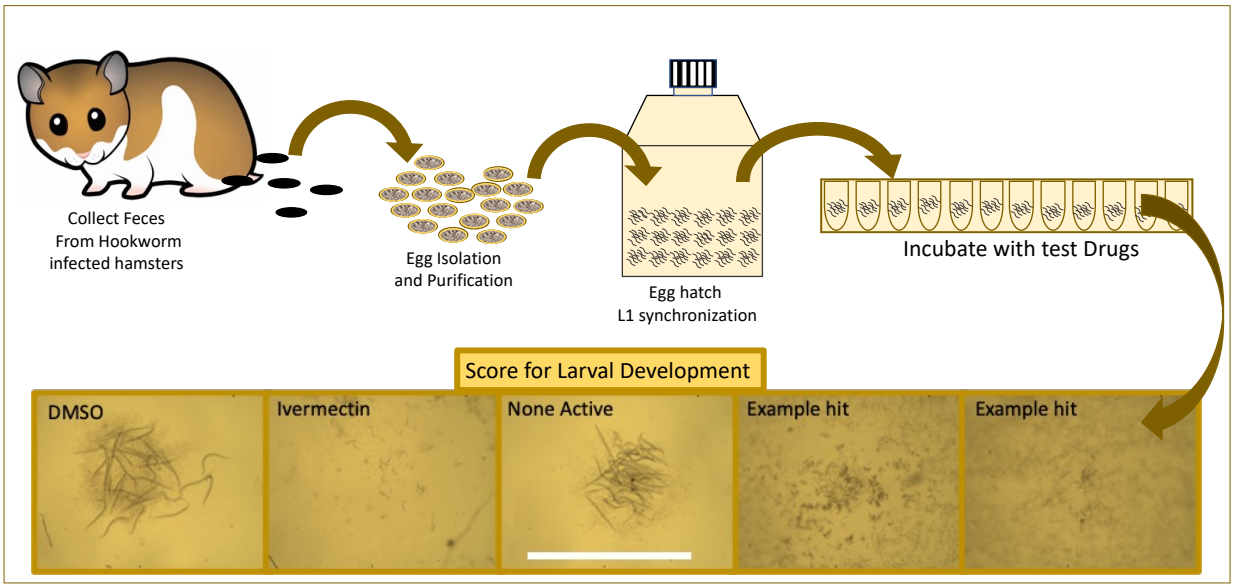

Figure 2: Primary screening using larval development of *A. ceylanicum* hookworms. To work around variability in egg hatch rates, we found that starting with the first larval stage led to more consistent and robust results (drugs that exclusively targeted egg hatch were very uncommon)[31]. Isolated and purified eggs were allowed to hatch in S-media into synchronized L1. Each 96-well assay plate includes four wells of ivermectin (10µM) as positive control and four wells of 0.25% DMSO as negative controls. After seven days of incubation at 28°C, assay plates were scored under a light microscope. The test compounds were considered active (hit) if at least 90% of all larvae in the well were killed as L1 or failed to develop. Scale bar 1 mm.

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Library	Unique Compounds	Screened doses µM	test wells screened in duplicate	*A. ceylanicum* L1 at 10µM (% hits)	*A. ceylanicum* adults 30µM (% hits)	*T. muris* Adults 30µM (% hits)
Life Chemical Diversity Set	15360	10	15360	491 (3.2)	33 (0.21)	7 (0.05)
Broad Institute REPO 1, 2 and 3	6743	10	6743	230 (3.4)	96 (1.42)	36 (0.53)
ICCB Longwood MOA	1245	10, 2, 0.4, 0.08	4980	65 (5.3)	17 (1.36)	9 (0.72)
ICCB Selleck Neuronal signaling	1031	10	1031	29 (2.8)	12 (1.16)	2 (0.19)
ICCB SYNthesis Kinase Inhibitor 2	96	10, 2, 0.4	288	8 (8.3)	1 (1.04)	1 (1.04)
ICCB EMD Kinase Inhibitor 1	244	10	244	10 (4.1)	2 (0.81)	2 (0.81)
ICCB ChemBridge Focused Kinase	250	10	250	2 (0.8)	0	0
ICCB ChemBridge Focused GPCR	250	10	250	2 (0.8)	0	0
ICCB LINCS 1 Kinase inhibitors	88	10, 2, 0.4, 0.08	352	4 (4.5)	2 (2.27)	1 (1.13)
ICCB LINCS 2 Kinase inhibitors	88	10, 2, 0.4, 0.08	352	2 (2.25)	0	0
ICCB LINCS 4 Kinase inhibitors	169	10, 2, 0.4, 0.08	676	8 (4.7)	3 (1.77)	3 (1.7)
SMSF Kinase Inhibitors	492	10	492	13 (3)	5 (1.01)	3 (0.69)
SMSF TEMTEC Natural Compounds	4240	10	4240	14 (0.33)	0	0
Total	**30238**		**38293**	**878 (2.9)**	**171 (0.56)**	**64 (0.21)**

Table 1: Screening summary of 30,238 compounds from 13 different libraries with information on screening doses, the number of hits per screening model, and the hit rates.

Hits from the Generic Diversity Set Library:

Of the 15,360 compounds in the generic diversity set tested against *A. ceylanicum* hookworm larvae, 491 were active, with a hit rate of 3.2%. These 491 larval hits were tested against the early adult stages of *A. ceylanicum* hookworm, yielding 33 hookworm adult-active compounds. An example of screening against the early adult stages of *A. ceylanicum* hookworms is shown in Figure 3. These 33 *A. ceylanicum* hookworm adult-active compounds were screened against *T. muris* adult whipworms, yielding seven hits active against the two evolutionarily divergent parasites. An example of screening against adult *T. muris* whipworms is shown in Figure 4.

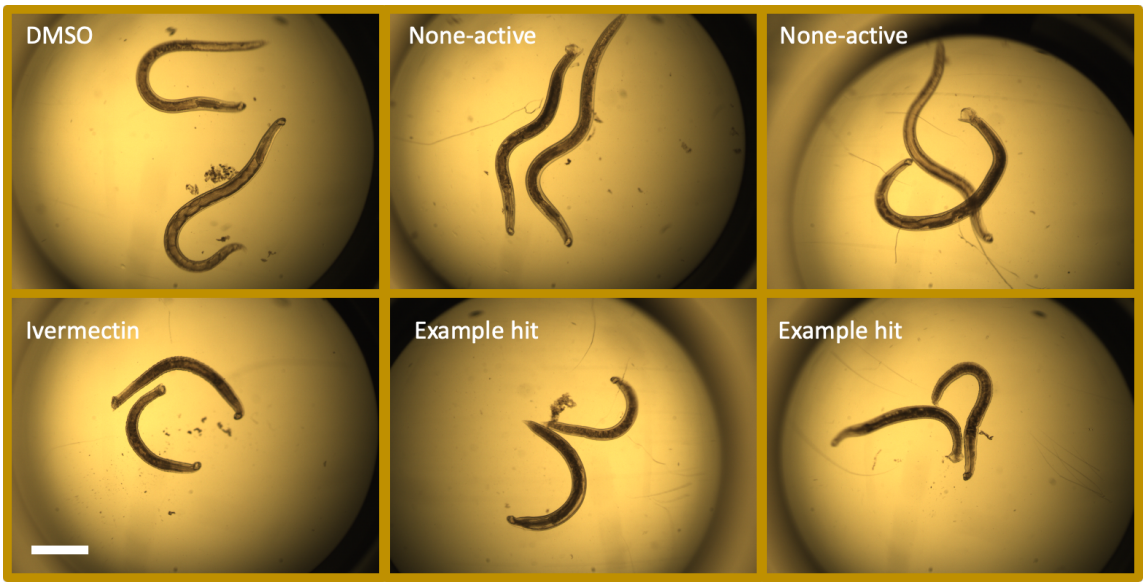

Figure 3. Young adult *A. ceylanicum* hookworm screens. The top panel shows healthy and motile worms from the DMSO-treated or nonactive test compounds. The lower panel shows dead or intoxicated worms from ivermectin or active compounds. Because young adult hookworms are much smaller than whipworms, we manually scored these versus using the Worminator. Scale bar 1 mm.

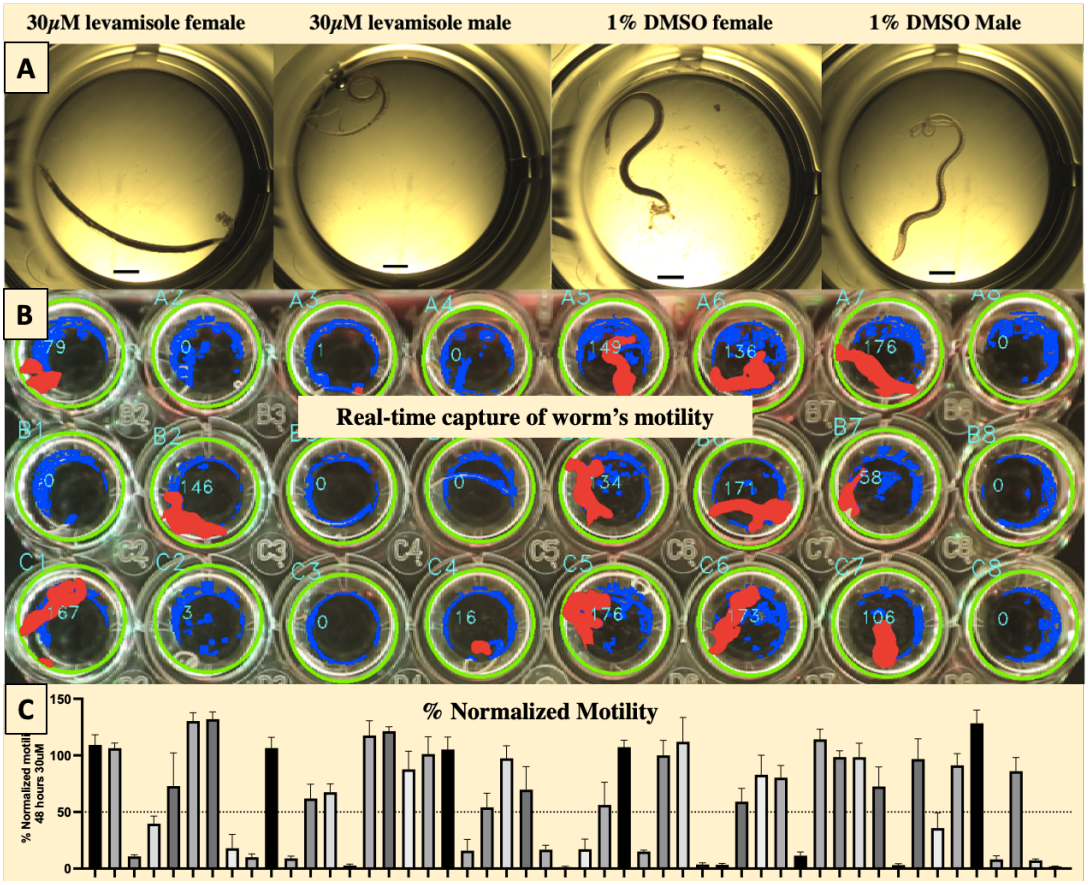

Figure 4: Adult parasite screening of *T. muris* whipworm**.** (A), Adult whipworms, male and female treated with 30μM Levamisole or 1% DMSO. Scale bar 1 mm. (B): Motility readout screenshot from Wormassay-6 software using the Womrinator imaging setup. (C). Example of calculated motility

readout, showing adult-parasite actives with Relative Motility (R.M.) less than 50% compared to the DMSO control.

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182 **Hits from the Repurposing and Mechanism of Action (MoA) Libraries**

183 Of the 6,743 compounds tested from the repurposing library, we identified 230 *A. ceylanicum*
184 hookworm larval-active compounds (hit rate of 3.4%), from which 96 showed activity against
185 adult *A. ceylanicum* hookworms (hit rate 1.42%). Of these 96 compounds, 36 were active
186 against adult *T. muris* whipworms with a final hit rate of 0.53%. Similarly, The ICCB-Longwood
187 MoA library contained 1,245 high-quality compounds with known mechanisms of action. We
188 identified 65 *A. ceylanicum* hookworm larval-active compounds with a 5.2% hit rate, the highest
189 among all tested libraries. Seventeen of these compounds carried adulticidal activity against *A.*
190 *ceylanicum* hookworms, from which nine were active against *T. muris* whipworm adults, with a
191 final hit rate of 0.72%.

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193 **Hits from the Neuronal and the GPCR libraries**:

194 Of the 1031 compounds with known modulation of neuronal signaling, we identified 29 *A.*
195 *ceylanicum* hookworm larval-active compounds (hit rate of 2.8%). Of these 29 compounds, 12
196 showed activity against adult *A. ceylanicum* hookworms (1.1% of the total), from which two
197 were active against *T. muris* whipworm adults with a final hit rate of 0.19%. We also screened
198 250 known GPCR inhibitors and identified two compounds with larval activity against *A.*
199 *ceylanicum;* however, these compounds did not show activity against the adult stages of
200 parasites. One explanation for the lack of success with this library is that these inhibitors were
201 tailored initially for selective binding to human GPCRs.

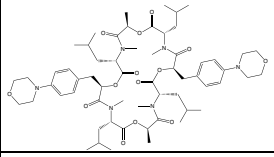
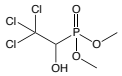
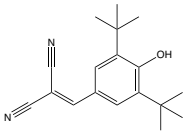
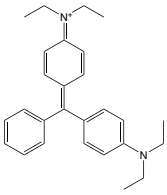
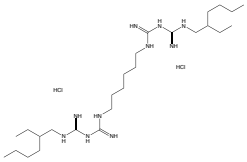
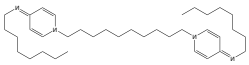
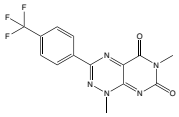
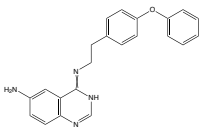
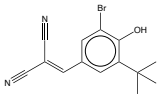
202

203 **Hits from the Kinase Inhibitor Libraries:**

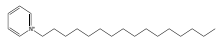
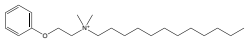
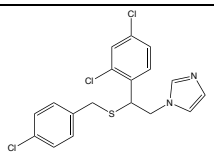
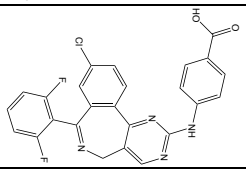
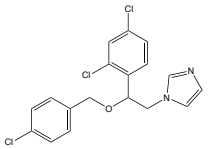
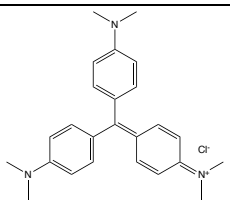
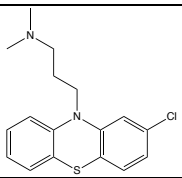
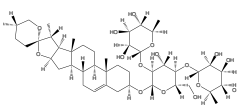
204 Of seven kinase inhibitor libraries with 1427 compounds (Table 1), we identified 47 with *A.*
205 *ceylanicum* larval activity, giving a hit rate of 3.2%. Of those 47 compounds, 13 showed
206 activities against the adult stages of *A. ceylanicum* hookworms (0.9%), of which 10 compounds
207 were also active against *T. muris* whipworm adults with a hit rate of 0.7%.
208
209 **Hits from the Natural Products Library**:
210 Of 4,240 purified compounds from natural products or derived from natural products, we
211 identified 14 compounds with *A. ceylanicum* larval activity, giving the lowest primary screen hit
212 rate of 0.33%. None of these 14 larvicidal compounds showed activity against the adult stages of
213 *A. ceylanicum* hookworms.
214
215 **Summary of 30,238 compound screen**
216 Collectively, we identified 878 compounds with *A. ceylanicum* larval activity, giving an overall
217 primary screen hit rate of 2.9% (Table 1). In our secondary screen, we tested all larval-active
218 compounds at 30 μM against the adult stages of *A. ceylanicum* hookworm parasites. Of those,
219 171 (19.5% of the larval hits) had anthelmintic activity toward adult hookworms, representing a
220 hit rate of 0.5% based on the total number of compounds in the initial libraries. These 171
221 hookworm-adult actives were tested against adult *T. muris* whipworms at 30 μM. Sixty-four
222 compounds (37% of the adult hookworm hits and 0.21% based on the total number of
223 compounds in all the libraries) showed activity against whipworm adults (Table 1). Of the 64
224 compounds identified from all libraries, nine were redundant (included independently in multiple
225 libraries), yielding 55 unique active compounds, listed in Table 2.
226

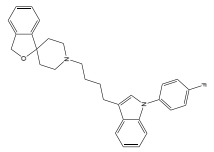
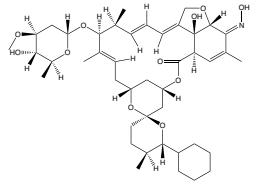
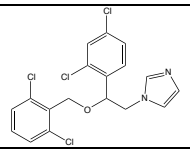
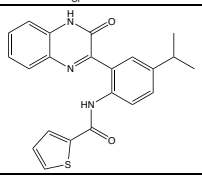
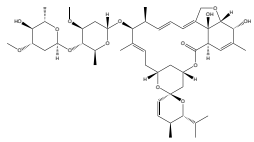
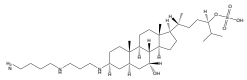
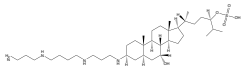
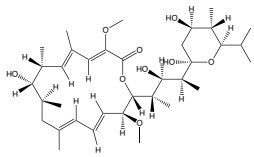
227 Screened libraries contained known anthelmintic compounds, and we successfully identified
228 albendazole, oxibendazole, flubendazole, methiazole, oxfendazole, thiabendazole, ivermectin,
229 abamectin, dormamectin, emamectine, eprinomectin, selamectin, emodepside, closantel,
230 monepantel, and moxidectin as part of the primary and secondary *A. ceylanicum* hookworm
231 screens. However, many of these anthelmintics didn't show activity in our tertiary screening
232 against the *T. muris* whipworms at 30µM, highlighting the whipworms' resilience to
233 anthelmintics compared to hookworms. For example, the published 50% inhibitory
234 concentrations (IC_{50}) of albendazole and ivermectin against *T. muris* whipworms are both >
235 200µg/ml[40]. Only anthelmintic compounds that showed activity at 30µM against *A. ceylanicum*
236 and *T. muris* are shown in Table 2.
237
238 **Data Mining**
239 The 55 broadly active compounds were subjected to a literature search to identify, if known,
240 their possible mode of action and the eukaryotic drug targets in other organisms and the
241 orthologous targets in phylum Nematoda, focusing on *C. elegans* in clade V and *T. muris* in
242 clade I (Table 2). The compounds encompass a wide range of putative target classes including
243 potassium channel, glutamate-gated chloride channel, acetylcholinesterase, acetylcholine
244 receptor, tyrosine kinases, aurora kinase, phosphatidylinositol 3-kinase (PI3K), tyrosine
245 phosphatase, insulin growth factor receptor (IGF1-R), nitric oxide synthase (NOS), Exchange
246 Protein Activated by Cyclic AMP (EPAC), pro-survival protein (BCL2), ATP synthase,
247 translation elongation factor, Enoyl-acyl carrier protein reductase (ENR), p38 mitogen-activated
248 protein Kinase (p38 MAPK), sphingosine 1-phosphate receptor (S1PR), Heat Shock Protein-90
249 (HSP-90), Dopamine D2 receptor, liver X receptor (LXR), serotonin (5-HT)3 receptor,

250 chemokine CXCR3 receptor, and ubiquitin specific peptidase 7 (USP-7) and Phosphatase
251 localized to Mitochondrion 1(PTPMT1). Compounds identified here with well-known targets
252 and mechanisms of action in other organisms may be used as probes for druggable and essential
253 ortholog targets in nematode parasites (Table 2). For example, alexidine was shown to induce
254 selective responses similar to the knockdown of its target PTPMT1[41]. Similarly, T0901317 is a
255 well-known selective agonist of LXR used in many studies to investigate the physiological
256 effects of LXR on cholesterol and lipid homeostasis; notably, T0901317 induction of LXR was
257 found to reduce cholesterol biosynthesis and to induce cholesterol breakdown into bile acids [42-44].
258 As examples, we discuss putative nematode targets of four of these-- BCL2, NOS, PTPMT1, and
259 LXRs, selected because these targets are known to be essential (See Supplementary Discussion
260 Part A).

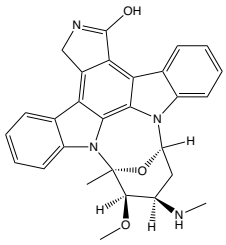
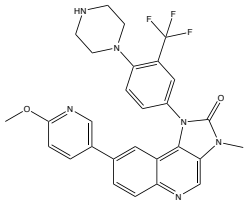
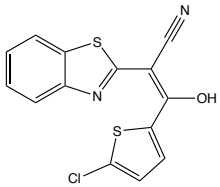
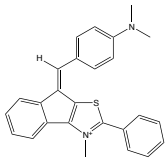
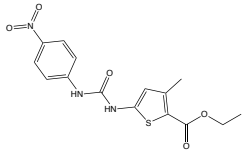
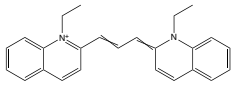
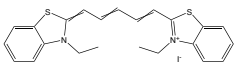
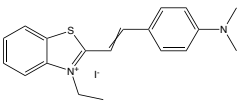
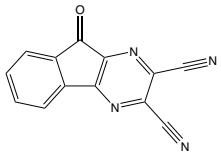
Drug Names	Structure	Source Library	HW M.I. 30µM	WW % R.M. 30µM	Known MoA /Target	Target orthologue in Nematodes
Emodepside		REPO	0	8.6	Anthelminthic, bind to nematode Latrophilin-like receptor (LAT-1), Voltage-gated activated potassium channels (SLO-1) [45, 46]	Lat-1 and slo-1in *C. elegans*, TMP05851 and TMP00134 in *T. muris*
Metrifonate; TRICHLORFON		REPO	0.75	1.3	Acetylcholinesterase (ACHE) inhibitor [47]	ace-1 in *C. elegans*, TMP09383 in *T. muris*
Tyrphostin-A9; Malonoben		REPO, SMSF kinase	0	9.1	Protein tyrosine kinase receptor inhibitor/ Epithelial Growth Factor Receptor (EGFR), Platelet-Derived Growth Factor Receptor (PDGFR) [48]	Let-23, kin-30, in *C. elegans*, TMP05029 and TMP12675 in *T. muris*
Brilliant-green		REPO	0	5.1	--	--
Alexidine		REPO	1.75	1.6	Protein Tyrosine Phosphatase localized to the Mitochondrion 1(PTPMT1) [49]	F28C6.8 in *C. elegans*, TM12441 in *T. muris*
Octenidine		REPO	0.75	7.3	--	--
Walrycin-B		REPO	0	8.6	--	--
EVP4593; QNZ		REPO, ICCB MOA, EMD kinase, LINCS Kinase	0	35.8	TNF-α and $NF_k\beta$ pathway inhibitor [50]	--
AG-1024		REPO	2	7.5	Protein tyrosine kinase receptor inhibitor/ Insulin growth factor receptor inhibitor/ (IGF1R) [51]	Daf-2 in *C. elegans*, TMP10632 in *T. muris*

Tyrphostin AG879		REPO, SMSF kinase	0	20	Protein tyrosine kinase receptor inhibitor/Nerve Growth Factor Receptor (NGFR) [48]	--
Diphenyleneiodonium		REPO	0	15.2	Nitric oxide synthase inhibitor/ NOS2, NOS3 [52]	Lacked in *C. elegans* NOS-Like in *A. suum*
ESI-09		REPO	0.5	16.2	Exchange protein activated by cAMP isoform 2 (EPAC2) inhibitor [53, 54]	epac-1 in *C. elegans*, TMP12712 in *T. muris*
Obatoclax		REPO, ICCB MOA	0.5	10.7	Induce apoptosis via the inhibition of the pro-survival protein/ BCL2[55]	ced-9 in *C. elegans*, TMP02363 in *T. muris*
Oligomycin-A		REPO	1.25	15.9	Mitochondrial ATP synthesis inhibitor/ ATP5A[56]	atp-1 in *C. elegans,* TMP09383 in *T. muris*
Pyrithione-zinc		REPO	0	3.6	KCNQ Kv7 potassium channels agonist [57]	kqt-1 in *C. elegans, TMP05091 in T. muris*
NH125		REPO, LINCS 1 kinase	1.25	8	Eukaryotic translation elongation factor 2 kinase (EEF2K) inhibitor [58]	efk-1 in *C. elegans* and TMP13626 in *T. muris*
Tetramisole		REPO	2.5	9.4	Anthelminthic, acetylcholine receptor agonist [59]	acetylcholine receptor agonist
Levamisole		REPO	1	10.1	Anthelminthic, acetylcholine receptor agonist [59]	acetylcholine receptor agonist
Triclocarban		REPO	0	12.7	--	--
Semapimod		REPO	1.75	39.7	Inhibit Toll Like Receptors (TLR) signaling by targeting TLRs chaperone gp96 [60]	*enpl-1* in *C. elegans* and TMP11927 in *T. muris*
Amiselimod		REPO	1	3.3	Sphingosine 1-phosphate receptor (S1PR) modulator [61]	Spin-2 in *C. elegans* and

						TMP13635 in *T. muris*
Cetylpyridinium		REPO	0.25	15.1	HSP-90 inhibitor [62]	Hsp-90 in *C. elegans*, TMP11927 in *T. muris*
Domiphen		REPO	0	11.5	Blockade of human ether-a-go-go related gene (HERG) potassium channels [63]	unc-103 in *C. elegans*, TMP12566 in *T. muris*
Sulconazole		REPO	1	3.1	Sterol 14 alpha Demethylase cytochrome (CYP-51) inhibitor [64]	--
MLN8054		REPO	1.5	18	Aurora Kinase inhibitor/ AURKA [65]	Air-2 in *C. elegans*, TMP01390 in *T. muris*
Cetrimonium		REPO	0.25	2.7	--	--
Econazole		REPO	1.2	7.6	Sterol 14 alpha Demethylase cytochrome (CYP-51) inhibitor [64]	--
Crystal-violet		REPO	0.25	5.3	HSP-90 inhibitor [62]	Hsp-90 in C. elegans TMP11927 in *T. muris*
Chlorpromazine		REPO	0.2	2.7	Dopamine D2 receptor antagonist [66]	dop-2 and dop-3 in C. elegans and TMP13188 in *T. muris*
Dioscin		REPO	1.5	15.3	Antagonize chemokine receptor CXCR3 block interaction to IP-10 cytokine [67]	Npr-20 in *C. elegans,* TMP12387 in *T. muris*

Siramesine; Lu28-179		REPO	2	2.1	σ_2R receptor agonist [68]	--
Selamectin		REPO	1	17.1	Anthelminthic, Neuromuscular paralysis via activation of Glutamate-gated chloride channels [69-71]	avr-14 in *C. elegans*, TMP04454 in *T. muris*
Isoconazole		REPO	0.4	3.1	Sterol 14 alpha Demethylase cytochrome (CYP-51) inhibitor [64]	--
ML281		REPO	1.5	16.7	Serine/threonine kinase inhibitor/ STK33[72]	--
Abamectin		REPO	0	13.1	Anthelminthic, Neuromuscular paralysis via activation of Glutamate-gated chloride channels [69-71]	avr-14 in *C. elegans*, TMP04454 in *T. muris*
Squalamine		REPO	1	3.1	protein tyrosine phosphatase (ptpn1b) inhibitor [73]	Sdf-1in *C. elegans*, TMP05082 in *T. muris*
Trodusquemine		REPO	0.25	2.1	protein tyrosine phosphatase (ptpn1b) inhibitor[73]	Sdf-1in C. elegans, TMP05082 in *T. muris*
Bafilomycin A1		ICCB MOA	0.5	52.7	Disrupt Autophagic flux by inhibiting V-ATPase and Ca-P60A/SERCA[74]	Vha-19 and sca-1 in *C. elegans*, TMP10618 and TMP09741 in *T. muris*
NMS-873		ICCB MOA	0	34.5	Allosteric inhibitor of the ATPase	cdc-48 in *C. elegans,*

					valosin-containing protein (VCP/p97) [75]	TMP02658 in *T. muris*
1-Phenyl-3-[4-(trifluoromethoxy) phenyl]urea		ICCB MOA	0	50.2	--	--
2,3-Diphenyl-5-[(2-methoxyphenyl)amino]-1,2,4-thiadiazole-2-ium		ICCB MOA	0	41.7	--	--
Furan-2-ylmethyl-(5-phenyl-thieno[2,3-d]pyrimidin-4-yl)-amine		ICCB MOA	0	41.9	--	--
4-Bromo-N-[2,2,2-trichloro-1-(4-chloroanilino)ethyl]benzamide; AMG7160		ICCB MOA	2	27.6	Ca^{2+} Cation channel, transient receptor potential ankyrin 1 (TRPA1) antagonist [76]	Trpa-1 in *C. elegans,* TMP11515 in *T. muris*
T0901317		Selleck Neuronal	2	37.6	Liver X receptor LXR (Nr1H2, Nr1H3) agonist [42-44, 77]	nhr-247 and nhr-8 in *C. elegans*, TMP09010 and TMP06463 in *T. muris*
Vortioxetine; Lu AA21004		Selleck Neuronal	2	30.9	5-HT3-R antagonist [78, 79] and serotonin transporter (SERT) inhibitor [80]	Lgc-7, lgc-8 *and mod-5* in *C. elegans,* TMP06220, TMP00636 and TMP10599 in *T. muris*
Sorafenib		SMSF, SYNthesis2, LINCS 1 kinase	0	20.6	Inhibit Raf-1, BRAF (serine/threonine Kinase), VEGFR, PDGFR, KIT, FLT3, FGFR1(tyrosine kinase) [81]	Lin-45, ver-1, ver-4, kin-30, old-2 and igcm-4 in *C. elegans* TMP03531, TMP12675, TMP13825, TMP00128, TMP02580, TMP02073 in *T. muris*

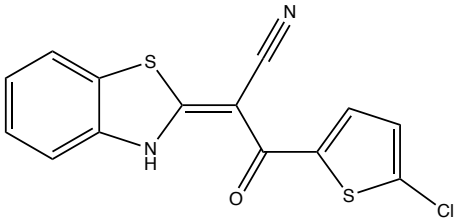
Staurosporine		EMD Kinase	1.25	27.7	Promiscuous Protein Kinase inhibitor [82]	
NVP-BGT226; HMSL10349-106		LINCS 1 Kinase	1.5	0	phosphatidylinositol 3-kinase (PI3K) inhibitor and MTOR [83]	age-1 in *C. elegans*, TMP06498 in *T. muris*
F0317-0202		Diversity set	0	10.7	--	--
F1059-0041		Diversity set	1.75	19.5	--	--
F1620-0033		Diversity set	1	13	--	--
F9995-0076		Diversity set	0	3.7	--	--
F9995-0233; dithiazanine iodide		Diversity set	0	1.75	Anthelminthic activity via inhibition of oxygen consumption and interreference with glucose uptake [84-88].	--
F0900-4647		Diversity set	0	3	--	--
F1345-0623; HBX		Diversity set	0.5	9	USP7 inhibitor [89]	math-33 in *C. elegans*, TMP04955 in *T. muris*

261 **Table 2:** List of 55 broadly active compounds including chemical structure, source library, *in vitro* activity against
262 *A. ceylanicum* hookworms (HW motility index (M.I.) where 3 is healthy and 0 is completely immotile), *in vitro*
263 activity against *T. muris* whipworms (WW percent Relative Motility (% R.M.),100% is healthy; 0% is non-motile)
264 to the DMSO control, known mode of action in eukaryotic organisms, and the orthologous targets in nematodes if
265 any. (--) no known target.
266
267 **Novel anthelmintic scaffold:**

268 We identified seven compounds with broad anthelmintic activity from the Life Chemical
269 Diversity Set, which is compiled of *de novo* and newly synthesized molecules developed for
270 initial phenotypic screening. Among these seven actives from this library of novel scaffolds, we
271 identified F0317-0202 as a potential lead for further studies, given its better drug-like structure
272 over the other six. To further investigate, 28 analogs of F0317-0202 were identified using
273 structural similarity online searching tools and purchased from a commercial vendor (Life
274 Chemical), which could serve as training sets for ligand-based optimization and generate the
275 SAR model. We screened this set of analogs against adult *T. muris* whipworms, followed by
276 older and larger *A. ceylanicum* hookworm adults, which could read more easily in the
277 Worminator.
278
279 Of the 28 analogs, we identified 12 compounds (41%) that inhibit *T. muris* whipworms motility
280 by ≥ 50% relative to the DMSO control (Figure 5, Supplementary Figure 1). All 12 were tested
281 and found to be active against *A. ceylanicum* hookworm adults (Figure 5 and Supplementary
282 Figure 1). Of these 12 broadly active compounds, four (F0317-0013, F0317-0161, F0328-0210,
283 and F0317-0009), along with the parent, showed near-complete motility inhibition at 30µM
284 against the two parasites. Conversely, of the 28 analogs, 16 showed significantly reduced/no
285 activity against whipworm adults.
286

Based on these data, an F0317-0202 SAR model for potent and broad anthelmintic activity was built and found to be quite strict— slight modifications could abolish the anthelmintic activity entirely. The functional core identified in this potent scaffold was the 1,3-benzothiazol-2-ylidene-3-oxopentanenitrile (Figure 6A) linked to an aromatic ring (phenol or thiophene) with one of the following electrons withdrawing group replacements: chlorine, bromine, nitrile or 3,5 dimethylpiperidine-1-sulfonyl as indicated in Figure 6B.

F0317-0202



HW R.M. (30µM): 1%
WW R.M. (30µM): 0%

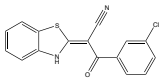
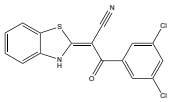
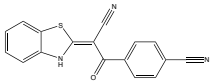
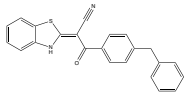
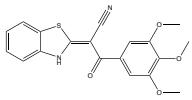
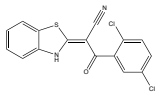
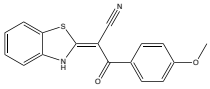
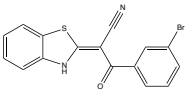
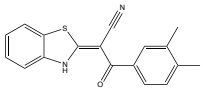
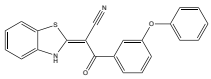
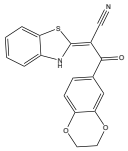
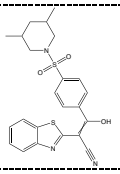
F0317-0019	F0317-0013*	F0317-0161*	F0336-0103	F0317-0175	F0328-0210*
					
HW R.M.: 2% WW R.M.: 22%	HW R.M: 3% WWR.M: 0%	HW R.M.: 1% WW R.M.: 2%	HW R.M.: 1% WW R.M.: 50%	HW R.M.: 1% WW R.M.: 37%	HW R.M.: 1% WW R.M.: 8%
F0317-0012	F0317-0009*	F0328-0550	F0317-0160	F0882-0289	F0463-0179
					
HW R.M.: 2% WW R.M.: 16%	HW R.M.: 2% WW R.M.: 1%	HW R.M.: 1% WW R.M.: 20%	HW R.M.: 6% WW R.M.: 44%	HW R.M.: 11% WW R.M.: 42%	HW R.M.: 33% WW R.M.: 1%

Figure 5: F0317-0202 broadly active analogs: Chemical structure of the top tested analogs and the % of relative motility to the DMSO control (R.M.) of 30µM relative to DMSO the *in vitro* activity, against *A. ceylanicum* hookworms (HW) and *T. muris* whipworms (WW).

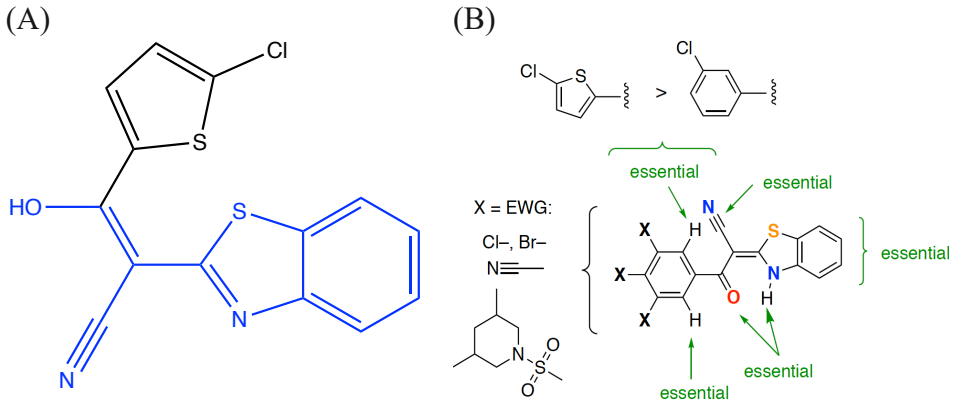


Figure 6: F0317-0202 Structure-activity relationship (SAR) model. (A) The bioactive group in F0317-0202 is 1,3-benzothiazol-2-ylidene-3-oxopentanenitrile (blue). (B) Structure-activity relationship (SAR) model generated for F0317-0202.

Discussion

Human GINs are among the most prevalent infections of the NTDs. Because understanding of these parasites' basic biology is still limited, whole organism screening (*i.e.*, phenotypic screening vs. target-based screening) remains the most effective approach in finding effective anthelmintics. We previously validated a novel screening pipeline using a set of 1280 FDA-approved compounds from a single library [31]. This screening pipeline utilizes two phylogenetically diverse nematode parasites, *A. ceylanicum* hookworms and *T. muris* whipworms, located at opposite ends of the phylum Nematoda phylogenetic tree and that are either a human parasite (*A. ceylanicum*) or closely related to a human parasite (*T. muris* vs *T. trichiura*). By selecting these diverse nematodes, we aim to identify compounds with broad anthelmintic activity. Here, using much larger and more diverse sets of compound libraries, we used our pipeline to screen more than 30,000 compounds, which is unprecedented for targeting the GINs of humans.

The life chemical diversity set library comprises 15,360 compounds representing 1,717 chemical scaffolds from novel and newly synthesized small molecules. Using our screening pipeline, we uncovered seven molecules with broad activity against parasitic nematodes, giving a hit rate of

315 0.045%. Among these seven, four are polymethine cyanine dyes: F1059-0041, F9995-0076,
316 F0900-4647, and dithiazanine iodide (F9995-0233). Dithiazanine iodide is known to be active
317 against hookworms, threadworms, roundworms, pinworms, and whipworms [84-88], providing
318 additional validation for our pipeline. Of the other three non-polymethine cyanine dyes, F1345-
319 0623 has been identified as a potential inhibitor (IC_{50} in the sub-micromolar range) of the
320 deubiquitinase USP7 [89]; homologous proteins have been found to play essential roles in *C.*
321 *elegans* (math-33)[90] and *Schistosoma mansoni* (SmUSP-7) [91].
322
323 Another compound, F0317-0202, has an excellent drug-like structure, and was selected for
324 further studies. F0317-0202 includes two aromatic ring systems (thiophene and benzothiazole),
325 listed among the most frequently used ring systems from small molecules in the FDA Orange
326 book [92]. The compound also has two functional groups (OH and CN), listed among the most
327 frequent functional groups occurring in bioactive molecules [93]. The compound's
328 physicochemical property agrees with Lipinski's rule of five for drug-likeness, with a molecular
329 weight of 318.8g/mol (less than 500), one hydrogen bond donor (less than five), five hydrogen
330 bond acceptor (less than 10), and lipophilicity score (Log P) of 4.3 (less than five)[94]. Out of 28
331 tested analogs of F0317-0202, 12 compounds (42%) showed potent activity against whipworm
332 and hookworm parasites, with four compounds showing near-complete inhibition in motility in
333 the two parasites. We consider these five novel compounds (F0317-0202 and four of its analogs,
334 F0317-0013, F0317-0161, F0328-0210, and F0317-0009) as well-positioned for further *in vitro*
335 characterization, lead optimization, and *in vivo* validation.
336

337 In contrast to screening diversity sets or random compound collections, we also screened
338 repurposing libraries, which contain compounds already approved or associated with clinical
339 trials and which can serve as a source for novel therapeutics for new indications [95]. The Broad
340 Institute repurposing library, which we used in our screen, contains 6,743 compounds from
341 molecules with high structural diversity and with 2006 known protein targets, including but not
342 limited to the G protein-coupled receptors (GPCR), acetylcholine receptors, protein kinases,
343 oxygenases, voltage-gated channels, and nuclear hormone receptors[96]. In addition, screening
344 compounds with known targets can be highly beneficial, especially against NTDs where a deep
345 understanding of their biology is still lacking. Compounds with known modes of action offer a
346 significant advantage in drug development because they can act as probes for essential targets,
347 assuming the targets are known. For this reason, we also screened the ICCB-Longwood
348 mechanism of action library containing 1,245 high-quality compounds with known targets.
349
350 The total number of compounds tested from both libraries was 7,988, uncovering 45 compounds
351 with putative broad activity against parasitic nematodes, giving a hit rate of 0.56%, 12.4 times
352 that obtained from the generic diversity set library. These results are the first to thoroughly
353 compare a generic dissimilarity-based library type with repurposing/known mode of action
354 compounds against parasitic nematodes using the same screening conditions and pipeline. Our
355 results indicate that repurposing/known mode of action libraries are significantly more (>10X;
356 $P < 0.00001$ Fisher's exact test) likely to uncover active compounds.
357
358 The plethora of the chemical universe is expected to contain 10^{12} to 10^{180} drug-like compounds[97],
359 which are impossible to screen against any single organism, not to mention GINs. Therefore,

360 techniques and strategies to design chemical libraries covering essential pharmacological targets
361 are highly recommended. Among these approaches is the compilation of a target-focused library
362 of compounds, which can be fitted by molecular docking to bind and inhibit the function of a
363 specific target or protein family[98, 99]. This strategy has been previously validated using a kinase-
364 targeted library of 1440 compounds tailored to fit in a total of 41 kinases from five different
365 families, showing a 6.7-fold increase in the overall hits compared to the generic collection of
366 molecules[100]. Target-focused screening improves efficiency by excluding compounds unlikely to
367 modulate known target proteins of interest and is associated with increased potency and
368 specificity[101].
369
370 We found that target-based libraries could serve as good sources for anthelmintic discovery. We
371 screened nine target-focused libraries, including one for neuronal targets, one for GPCRs, and
372 seven libraries targeting protein kinases. Neuronal signaling and the neuromuscular system are
373 essential for nematodes' survival, including feeding, mating, egg laying, and locomotion; thus,
374 many anthelmintics drugs target the nematode nervous system[102]. Neuropeptide GPCRs have
375 been identified as potential and druggable targets and were found to play critical roles in the
376 helminth neuromuscular function[103]. Kinases are the second most targeted protein class after
377 GPCRs, with over 200 kinase inhibitors having reached the clinical phases and 52 FDA-
378 approved drugs targeting protein kinases[36, 104].
379
380 Together, these nine libraries contain 2,708 compounds, and within these, we identified 12
381 compounds with putative broad activity, giving a hit rate of 0.44%, statistically the same as that
382 obtained from the combined repurposing and mode of action libraries (P=0.54, Fisher's exact

383 test). It is worth noting that three of the nine target-based libraries resulted in no hits with activity
384 against hookworms and whipworms: the ChemBridge Focused kinase, GPCR, and the LINCS 2
385 kinase inhibitors, which could be explained by the lack of specificity to the parasitic kinases and
386 GPCRs, as these libraries were fitted to selectively binding specific human targets.
387
388 Historically, natural products have been the primary source of remedies for human ailments, and
389 purified natural molecules or direct derivatives have contributed to at least 34% of all drugs
390 approved by the FDA between 1981 and 2010[105]. Using our novel pipeline, we screened the
391 TimTec library of 4,240 purified compounds from natural products or direct derivatives and
392 identified 14 compounds with *A. ceylanicum* larval activity. However, none of these larvicidal
393 compounds carried activity against adult parasites. This result reflects may reflect biases of this
394 particular library.
395
396 In summary, we have developed a novel high throughput screening pipeline to identify
397 anthelmintic compounds for GIN parasites and screened 13 different libraries with a total of
398 30,238 compounds that led to the discovery of 55 compounds with potent activity against two
399 species of evolutionary distant nematode parasites. Repurposing, mode of action, and target-
400 based libraries were more profitable than the diversity set for anthelmintic screening. For these
401 55 compounds, we reported the known modes of action and related targets in other organisms,
402 and potential counterparts in the nematode, if available. As an example, we highlighted four
403 nematode targets as potential candidates for target-based drug screening. We also identified
404 thiazolyl oxopropanenitrile as a novel chemical scaffold with broader anthelmintic activity
405 identified from the diversity set library and established the SAR models for potent activity

406 against hookworms and whipworms. Our findings open the doors for multiple follow-up
407 approaches, including target identification, target-based screening, and ligand or structure-based
408 pharmacophore optimization for developing safe and parasite-selective molecules.
409
410 **Materials and Methods:**
411 **Ethics statement:**
412 All experiments involving animals were conducted per the recommendation of the National
413 Institute of Health (USA) Guide for Care and Use of Laboratory Animals and the Animal
414 Welfare Act. The animal protocols used in this study (PROTO202000044 and
415 PROTO202000071) were approved by the Institutional Animal Care and Use Committee
416 (IACUC) of UMASS Chan Medical School.
417
418 **Parasite maintenance in laboratory animals:** Following standard protocols, *A. ceylanicum*
419 hookworm parasites were maintained in golden Syrian hamsters[106], and *T. muris* whipworm
420 parasites were maintained in STAT6-/- mice [107].
421
422 **Primary screening using Larval Developmental Assay**:
423 Feces from *A. ceylanicum*-infected hamsters were collected ~18 days post-infection and about 20
424 grams of feces were processed for egg isolation and purification following salt/sugar flotation
425 protocol[108, 109]. Purified and surface sterilized eggs were incubated in S-medium[110] at 28 °C,
426 without any food source for about 20 hours, allowing eggs to hatch into synchronized first-stage
427 larvae [109]. Newly hatched L1 were washed three times in a S-medium. About 30-35 L1 larvae
428 were dispensed into each well of 96-well assay plates containing 90µL of S-media supplemented

429 with 100U/ml of penicillin, 100µg/ml streptomycin, and 2.5µg/ml amphotericin, *Escherichia coli*
430 OP50 with final OD600 of 0.3 and 0.15µl Dimethylsulfoxide (DMSO). Library plates were
431 diluted in S-media to 100µM and 1%DMSO. Using a multichannel pipette, 10µL of the library
432 plates were transferred into each well of the assay plates, giving a final concentration of 10µM of
433 test drug and 0.25% DMSO. Plates were prepared in duplicates. Test plates were incubated at 28
434 °C for seven days and then scored for development under a dissecting microscope. Test drugs
435 were considered hit if > 90% of L1 larvae failed to develop in duplicate wells. Each assay plate
436 includes four wells of 10µM ivermectin as positive control and four wells of 0.25% DMSO as
437 negative control.
438
439 **Secondary screening using young adults of hookworm parasites**:
440 We next screened hits from the primary screening (larval development) against the adult stages
441 of hookworm parasites. *A. ceylanicum* hookworm young adults were harvested 10-11 days post-
442 inoculation from the small intestines of infected hamsters. Harvested worms were washed and
443 incubated in prewarmed hookworm culture medium (RPMI1640 of pH 7.2 supplemented with
444 10% fetal bovine serum, 100U penicillin, 100µg/ml streptomycin, 2.5µg/ml amphotericin). Two
445 adults of *A. ceylanicum* hookworm were manually picked into each well of the 96-well assay
446 plates, containing 70µL of hookworm medium supplemented with 0.7µl DMSO. Using a
447 multichannel pipette, 30µL from the library plates diluted in hookworm medium to a final
448 concentration of 100µM and 1% DMSO were dispensed into screening wells, giving a final
449 concentration in assay plates of 30µM of test compounds and 1% DMSO with duplicate
450 wells/drug. Assay plates were incubated for 48 hours at 37 °C and 5% CO_2. Drug activity was
451 determined using the standard motility index [31, 107]. A motility index of 3 was given to vigorous

452 healthy adults, 2 for motile but slow adults, 1 for immotile adults that moved after stimulation by
453 touching, and 0 for non-motile adults even after touching. The drug was considered a hit if the
454 motility index of at least three of the four *A. ceylanicum* hookworm adults were scored ≤1 and/or
455 if all the parasites in the well had significant morphological defects (*e.g.*, shrunken cuticles,
456 darkened intestines (Figure 3). Each assay plate included four positive control wells (30µM
457 ivermectin) and four negative control wells (1% DMSO).
458
459 **Tertiary screening using adult whipworm parasites**:
460 We next screened *A. ceylanicum* hookworm adult hits against *T. muris* whipworm parasites. *T.*
461 *muris* adult whipworms were harvested from the cecum and large intestines of infected STAT6 -
462 /- mice. Single worms were manually picked into each well of a 48-well plate, six wells/ test
463 drug, and a final volume of 291µL of whipworm culture medium with 2.1µl DMSO (like
464 hookworm culture medium but supplemented with 5% fetal bovine serum). 9µL from library
465 plates diluted in whipworm medium to a final concentration of 1mM and 10% DMSO were
466 dispensed into each well of the screening plates, giving a final concentration of 30µM test drug
467 and 1% DMSO. Assay plates were incubated at 37 °C, and 5% CO_2. *T. muris* whipworm
468 motility was measured using an in-house assembled worminator and worm assay software[111] for
469 3 minutes after 24 and 48 hours. Drug activity was determined using the percent of relative
470 motility compared to DMSO control. Drugs were considered active if the 24- and/or 48-hour
471 motility was reduced by at least 50% compared to negative control (Figure 4).
472
473 **Chemical Libraries Screened**
474 **Life Chemical 15K diversity set**:

475 Life Chemical pre-plated diversity set library was obtained from the Small Molecules Screening
476 Facility (SMSF), UMASS Chan Medical School. The library contains 15,360 compounds
477 representing 1,717 chemical scaffolds with an average Tanimoto similarity index of 0.409.
478 Library compounds were plated in 96-well plates with 1ul of 10mM stock concentration. Hits
479 from the primary and secondary screening were cherrypicked into 96-well plates for *A.*
480 *ceylanicum* hookworms or *T. muris* whipworms screening.
481
482 **Broad Institute Repurposing Hub:**
483 The drug repurposing hub includes 6,743 compounds which were from a curated and annotated
484 collection of preclinical drugs in phase 1 (REPO 1) or phases 2-3 (REPO 2) and FDA-approved
485 and launched drugs (REPO 3). REPO drugs cover a wide range of disease areas, including but
486 not limited to infectious diseases, allergies, cardiology, dermatology, endocrinology,
487 metabolism, neurology, and gastroenterology. The library covers 1,133 different modes of action
488 and 2,183 different drug targets[96].
489
490 **ICCB Screening libraries**:
491 The Institute of Chemistry and Cell Biology-Longwood (ICCB-L) Screening Program at Harvard
492 Medical School offers a wide range of well-annotated drug screening libraries. We have screened
493 multiple ICCB-L libraries, including mechanism of action, neuronal targets, GPCR-based
494 inhibitors, and six libraries of kinase inhibitors.
495
496 *ICCB-L MOA*: the mechanism of the action library is a collection of high-quality annotated
497 compounds with biological activity against known targets with target-compound pairs based on

498 high target specificity and potency. The library contains 1,245 unique compounds, each plated in
499 4 doses. *ICCB-L Selleck Neuronal Targets:* the neuronal targets library was initially composed of
500 Selleck chemicals from FDA-approved compound sets that were determined to have biological
501 activity in neurologic research and neurological assays. *ICCB-L ChemBridge focused GPCR:*
502 This focused library of 250 GPCR inhibitors was selected from 13,000 GPCR inhibitors
503 assembled by ChemBridge. The library has 11 different scaffolds, each containing 19-24
504 compounds.
505
506 *Kinase inhibitor libraries:* in total, we screened focused sets of 1428 kinase inhibitors from 7
507 different kinase libraries, some of which were screened at multiple doses.
508 *ICCB-L SYNthesis kinase inhibitor:* this library was assembled by SYNthesis Med Chem of 96
509 compounds known to inhibit different kinases. *ICCB-L EMD Kinase inhibitors:* this library was
510 built by EMD of 244 protein kinase inhibitors. *ICCB-L ChemBridge Focused Kinase inhibitors:*
511 This library of 250 compounds was created and selected from the ChemBridge kinase-biased
512 library of 6000 compounds. These compounds were selected based on the pharmacophore
513 properties of known kinase inhibitors or from compounds predicted to interact with the ATP
514 binding site of kinases. In total, these compounds represent 16 different scaffolds that cover 34
515 kinase targets. *ICCB-L LINCS 1,2 and 4 Kinase inhibitors:* these three libraries were assembled
516 by LINCS together, including 345 compounds with known or predicted kinase inhibitors.
517
518 **SMSF kinase inhibitor library:** This library includes 429 kinase inhibitors with activity and
519 safety confirmed by preclinical research and clinical trials.
520

521 **Natural Products:** we screened the TimTec library of 4240 purified compounds from natural
522 products or derivatives. The TimTec library was obtained from the SMSF and includes two types
523 of compounds: *Natural Products Library (NPL):* The NPL library includes 1200 purified natural
524 compounds from plant, bacteria, fungi, and animal sources. *Natural Derivatives Library (NDL):*
525 The NDL library includes 3040 semi-natural compounds derived from natural molecules or
526 synthetic compounds with natural-like structures.
527
528 **Structure-Activity Relationship (SAR) of anthelmintic hits:** using vendors' databases and
529 online tools, analogs of F0317-0202 were identified and purchased from Life Chemicals. Each
530 analog has slight structural modification compared to the parent molecule. These analogs were
531 tested against the adult stages of *T. muris* whipworm and *A. ceylanicum* hookworm parasites at
532 30µM and scored using the Worminator at 24 and 48 hours. Hence, *A. ceylanicum* hookworm
533 parasites used in testing F0317-0202 analogs for SAR model were mature adults harvested 18
534 days post-infection.
535
536 **Authors contributions:**
537 M.A.E and R.V.A., conceived, designed, supervised, and interpreted the experiments. M.A.E and
538 R.V.A. wrote the manuscript. M.A.E, Y.M.K., and E.G. performed the *in vitro* screening. M.A.E
539 and S.S. performed data mining and analyzed SAR results. P.R.T. and L.B. contributed libraries,
540 technical advice, and inputs on the manuscript. M.A.E. designed the graphic abstract. R.V.A.
541 secured funding.
542
543 **Supporting information:**

544 [A] Additional details for the potential nematode targets (PDF)

545 [B] Additional experimental details for F0317-0202 tested analogs, chemical structures, and

546 motility inhibition (PDF)

547

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552

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