Short Report: *In vitro* Screening of Compounds against Laboratory and Field Isolates of Human Hookworm Reveals Quantitative Differences in Anthelmintic Susceptibility

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Abstract. A panel of 80 compounds was screened for anthelmintic activity against a laboratory strain of *Ancylostoma ceylanicum* and field isolates of hookworm obtained from school children in the Kintampo North District of the Brong Ahafo Region of Ghana. Although the laboratory strain of *A. ceylanicum* was more susceptible to the compounds tested than the field isolates of hookworm, a twofold increase in compound concentration resulted in comparable egg hatch percent inhibition for select compounds. These data provide evidence that the efficacy of anthelmintic compounds may be species-dependent and that field and laboratory strains of hookworm differ in their sensitivities to the anthelmintics tested. These data also suggest that both compound concentration and hookworm species must be considered when screening to identify novel anthelmintic compounds.

Human hookworm disease results from infection by two genera of hookworms, *Ancylostoma* spp. and *Necator americanus*. Albendazole (ABZ) and mebendazole (MBZ) are the primary drugs used to treat hookworm infection. A recent meta-analysis and review of field-based studies have shown that single-dose treatment cure rates with ABZ and MBZ are low (72% and 15%, respectively).^{1,2} Compounding these treatment failure rates in humans is the well-documented emergence of drug resistance to ABZ and MBZ among parasitic nematodes of agricultural and veterinary importance.^{3–5} In light of this information, the development of novel anthelmintic chemotherapeutics is necessary.

Currently, the identification and development of novel anthelmintic compounds to cure hookworm infection rely on laboratory-based screening for inhibitors of egg hatching, larval motility and morphology, and/or adult worm survival.^{6–9} However, novel compounds identified using small animal models of hookworm infection are rarely screened for comparable activity against field isolates of human hookworms.^{10–16} Therefore, the correlation between the anthelmintic activity of compounds against laboratory and field isolates of human hookworms remains largely unknown.

Moreover, for those cases in which anthelmintic activity is investigated, the larvicidal, rather than ovicidal, properties of a compound are usually analyzed.^{17–20} Ovicidal activity is more frequently and most readily assayed in resource-limited field settings, where larval and adult hookworm-based assays are impractical tools for assessing anthelmintic activity.^{21–23} In this study, we investigated the anthelmintic activity of a panel of compounds against laboratory and field isolates of hookworm using a standard egg hatch assay (EHA).^{6,7} These data support the use of the EHA in field investigations for anthelmintic discovery and provide evidence for the continued development of the active compounds that we identified as novel anthelmintics.^{22,24,25}

In total, 80 compounds were tested for anthelmintic activity against laboratory and field isolates of human hookworm using the EHA; 41 of the compounds were analogs of furoxan, an oxadiazole 2-oxide with proven nematocidal activity against the laboratory strain of *A. ceylanicum*.²⁶ An additional 14 compounds were analogs of furoxan that had not been previously tested against a laboratory strain of hookworm. The furoxan analogs were synthesized at the National Institutes of Health Chemical Genomics Center as part of the Therapeutics for Rare and Neglected Diseases Program.²⁷ To increase the diversity of chemotypes tested, a wide range of compounds (25 in total) was selected to be screened. Some of these molecules were chosen based on their putative targets. As an example, ABT-263 (navitoclax; Abbott Laboratories, Abbott Park, IL) is a molecule that acts on the B-cell lymphoma 2 (Bcl-2) protein, a regulator of apoptotic activity and an important anticancer target. It was recently shown that a similar molecule, ABT-737 (referred to here as National Chemical Genomics Center [NCGC] 00249278-01), binds the Bcl-2 protein in *Schistosoma mansoni* and may promote parasite death.²⁸ The remaining compounds in the set were Food and Drug Administration (FDA)-approved anticancer and antimalarial drugs selected for their potential anthelmintic properties.

For screening against *A. ceylanicum*, pooled fecal samples were harvested from Golden Syrian hamsters after infection by oral gavage with L3 stage *A. ceylanicum* as previously described.^{8,29,30} Hookworm eggs were purified from feces using a density floatation method, and the mean number of eggs per milliliter was calculated. For screening against field isolates of hookworm, duplicate fecal samples were collected from 142 Ghanaian school-aged children selected from five communities previously identified as having a high prevalence of hookworm infection.³¹ Each sample was examined for the presence of hookworm eggs using the Kato–Katz fecal smear technique, and hookworm eggs from positive samples were purified as described above and pooled.³² Purified eggs were pipetted into 96-well plates (100 eggs per well) containing water followed by the addition of compound dissolved in dimethyl sulfoxide (DMSO). Every compound was tested in duplicate at a final concentration of either 100 or 200 μ M. EHAs were incubated for 24 hours at ambient temperature. Water and ABZ served as the negative and positive controls,

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TABLE 1

In vitro susceptibility of laboratory isolates of *A. ceylanicum* and field isolates of human hookworms to furoxan analogs using egg hatch assay

Compound ID number	Egg hatch inhibition (%)	
	Laboratory isolates*	Field isolates
NCGC00167928-01†	100.0	76.4
NCGC00167944-01†	100.0	67.2
NCGC00167942-01	100.0	39.0
NCGC00167920-01	100.0	16.9
NCGC00167922-01	99.4	25.8
NCGC00182103-01	99.2	1.8
NCGC00167919-01	99.2	19.1
NCGC00241748-01	98.9	0
NCGC00183324-01	98.8	21.8
NCGC00167932-01	97.9	7.4
NCGC00182106-01†	97.9	58.0
NCGC00167927-01†	97.8	58.2
NCGC00167935-01†	97.1	72.2
NCGC00168364-01	96.1	2.2
NCGC00167918-01	95.9	46.1
NCGC00167924-01	95.0	10.3
NCGC00242405-01	92.7	3.7
NCGC00168368-01	92.0	9.3
NCGC00094237-01†	90.2	56.1

Data shown for analogs exhibiting inhibition values above 90%.

*Percent values represent the mean of two independent screenings—one conducted before and one conducted after the field screening.

†Denotes all tested compounds that inhibited hatching of field isolates by more than 50%.

respectively. The numbers of larvae and unhatched eggs were counted by light microscopy, and percent egg hatch inhibition values were calculated as

$$\text{Egg hatch inhibition (\%)} = \frac{(\# \text{unhatched})}{(\# \text{unhatched} + \# \text{larvae})} \times 100.$$

Of 80 compounds assayed, 20 compounds inhibited the hatching of *A. ceylanicum* by > 90% at 100 μ M (Tables 1 and 2). When tested against hookworm field isolates at the same concentration, only eight compounds inhibited hatching by > 50%, and no compound exhibited > 86% inhibition of egg hatching. All compounds that inhibited egg hatching of hookworm field isolates by > 50% were found to be > 90% effective at inhibiting *A. ceylanicum* egg hatching. Increasing the compound concentration to 200 μ M resulted in four additional compounds achieving egg hatch inhibition values over 50% (Table 3). At 200 μ M, 5 of 78 compounds inhibited hatching of field isolates of hookworm eggs to an equal or greater extent compared with the laboratory *A. ceylanicum* strain.

All compounds that inhibited the hatching of hookworm field isolates also possessed high ovicidal activity against *A. ceylanicum*. These data indicate that *A. ceylanicum* is more

TABLE 2

Highest *in vitro* egg hatch inhibition values among NCGC compounds tested against the laboratory strain of *A. ceylanicum* and field isolates of human hookworms

Compound ID number	Egg hatch inhibition (%)	
	Laboratory isolates	Field isolates
PERIFOSINE	98.3	0
NCGC00247880-01	87.1	85.9
NCGC00159390-13	56.4	12.0
NCGC00249281-01	55.9	0
NCGC00186465-03	26.3	62.1

TABLE 3

Compounds exhibiting greater than 50% percent *in vitro* egg hatch inhibition against hookworm field isolates and a laboratory strain of *A. ceylanicum* at 200 μ M

Compound ID number	Egg hatch inhibition (%)		
	Field isolates (200 μ M)	Field isolates (100 μ M)	Laboratory isolates* (100 μ M)
NCGC00167928-01	100.0	76.4	100.0
NCGC00167918-01†	99.8	46.1	95.9
NCGC00247880-01	95.6	85.9	87.1
NCGC00167944-01	90.0	67.2	100.0
NCGC00167935-01	85.6	72.2	97.1
NCGC00167927-01	85.1	58.2	97.8
NCGC00167922-01†	81.5	25.8	99.4
NCGC00167942-01†	81.3	39.0	100.0
NCGC00186465-01	66.1	62.1	26.3
NCGC00247881-01†	61.8	39.8	49.2

*Percent values represent the mean of two independent screenings—one conducted before and one conducted after the field screening.

†Denotes all tested compounds with egg hatch inhibition values that increased from below to above 50% with the twofold increase in inhibitor concentration.

susceptible to the compounds evaluated than are hookworms isolated from field samples. These differences may be independent of both compound class and molecular target. In select cases, the doubling of drug concentration led to egg hatch inhibition values > 90%, which were equal to or greater than those values obtained using laboratory isolates. These results suggest that broadening the range of compound concentration when screening may increase the chances of identifying compounds that possess ovicidal activity against both field and laboratory isolates of hookworm.

The difference in compound activity against field isolates and *A. ceylanicum* in our study, as well as other studies, suggests that this response may be species-dependent.³³ Both species of hookworm possess distinct geographic distribution patterns. *N. americanus* is believed to be the predominant species in tropical sub-Saharan Africa, including Ghana, with a minority caused by *A. duodenale*.^{34–37} This difference may influence the impact of ABZ treatment of hookworm-infected individuals within this area.

The finding that field isolates (presumably *N. americanus*) possess a more robust tolerance to anthelmintic treatment is not without precedent. Multiple studies investigating hookworm susceptibility to anthelmintics have reported that lower drug concentrations are effective against *A. ceylanicum* compared with *N. americanus*.^{7,38–40} These observations are supported by our comparative studies of the half-maximal inhibitory concentration (IC₅₀) values for ABZ and pyrantel pamoate (Table 4). For both compounds, higher IC₅₀ values were recorded for field isolates of hookworm compared with *A. ceylanicum*. These data support the conclusion that a differential response to anthelmintics exists between these two hookworm populations.

TABLE 4

In vitro egg hatch assay IC₅₀ values for anthelmintics tested against a laboratory strain of *A. ceylanicum* and field isolates of human hookworms

Anthelmintic	IC ₅₀ value	
	Laboratory isolates	Field isolates
Albendazole	56 nM	1.11 μ M
Pyrantel Pamoate	1.57 μ M	29.5 μ M
Levamisole	1.07 μ M	—

In this study, we investigated the anthelmintic activity of 80 compounds against laboratory and field isolates of human hookworms. The data suggest that both the species of hookworm and the concentration of compounds assayed are fundamental considerations in the design of future field-based compound screenings for anthelmintic discovery. The findings of this work also support the rationale of using laboratory-based screening of compounds against *A. ceylanicum* to identify novel anthelmintics given these considerations.

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