Caenorhabditis elegans is a useful model for anthelmintic discovery

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Parasitic nematodes infect one quarter of the world's population and impact all humans through widespread infection of crops and livestock. Resistance to current anthelmintics has prompted the search for new drugs. Traditional screens that rely on parasitic worms are costly and labor intensive, and target-based approaches have failed to yield novel anthelmintics. Here, we present our screen of 67,012 compounds to identify those that kill the non-parasitic nematode *C. elegans*. We rescreened our hits in two parasitic nematode species and two vertebrate models, and identified 30 structurally distinct anthelmintic lead molecules. Genetic screens of 19 million *C. elegans* mutants reveal those nematicides for which the generation of resistance is and is not likely. We discovered the target of one lead with nematode specificity and nanomolar potency as complex II of the electron transport chain. This work establishes *C. elegans* as an effective and cost-efficient model system for anthelmintic discovery.

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Elucidating conserved genetic mechanisms of anthelmintic resistance using *Caenorhabditis* nematodes

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Many neglected tropical diseases (NTD) are caused by parasitic nematodes. NTD carry a disease burden nearly one-quarter that of HIV and half that of malaria. They also indirectly increase susceptibility to HIV, tuberculosis, and malaria, exacerbating health impacts in the developing world. Within the last twenty years, a massive campaign to administer anti-nematode (anthelmintic) drugs was initiated. Unfortunately, there are few anthelmintics and resistance is growing rapidly, prompting an urgent need to identify resistance genes and new drugs. In addition to this impact on human health, nematode-borne diseases of livestock and plants are major agricultural problems, resulting in severe economic losses. Effective future treatments of parasitic infections require knowledge of which genetic variants cause resistance to a particular drug. For these reasons, my laboratory is working to identify the genetic basis for how resistance to anthelmintics develops. These discoveries will allow physicians to tailor existing treatments to exploit the weaknesses found in parasites and will enable researchers to identify new drugs that act broadly against multiple species.

Mechanisms of nematode drug resistance have been poorly understood because the parasites that impact human society only grow inside hosts not amenable to laboratory research (*e.g.* ruminants and humans). Additionally, these species do not have genetic tools to make experimentation possible. Given those challenges, we use an innovative system to take advantage of the powerful model nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*, including cutting-edge molecular genetic techniques with a new massively scaled quantitative phenotyping pipeline to rapidly and accurately measure offspring production, growth rate, and muscle behaviors for hundreds of individuals. These new assays are sensitive to drug effects and allow for rapid genetic mapping approaches. Previous studies using *C. elegans* focused on the laboratory strain N2, which only represents one strain in the species. Our current approach takes advantage of hundreds of natural strains and recombinant strain collections to identify genes that naturally vary in response to these drugs. These experimental tools combined with molecular and statistical genetics allowed us to identify numerous quantitative trait loci for avermectins, benzimidazoles, and nicotinic acetylcholine agonist drugs. For example, we identified five significant loci in response to avermectin beyond the known glutamate-gated chloride channels previously implicated. Additionally, we found that, although the natural population varies in benzimidazole sensitivity because of *ben-1*, other loci have larger phenotypic effects. We will present these results from *C. elegans* and also preliminary mappings using the related species *C. briggsae* to elucidate inter-species conservation of anthelmintic resistance.

The genome of *Ancylostoma ceylanicum*: distinguishing possible immunological decoys from possible drug and vaccine targets in a model hookworm

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Hookworms infect over 400 million people, stunting and impoverishing them. Unlike other hookworms, *Ancylostoma ceylanicum* infects both humans and other mammals, providing a laboratory model for hookworm disease. In order to distinguish between genes whose products may block the host immune system and those whose products might be drug or vaccine targets, we determined an *A. ceylanicum* genome sequence of 313 Mb, with transcriptomic data throughout infection showing expression of 30,738 genes. ~900 genes were upregulated during early infection in vivo, including ASPRs, a cryptic subfamily of Activation-associated Secreted Proteins (ASPs). ASPR genes are also present in the related intestinal parasites *Necator americanus*, *Oesophagostomum dentatum*, and *Heligmosomoides polygyrus* (*bakeri*), but not the trichostrongylid parasite *Haemonchus contortus*. Genes downregulated during early infection include ion channels, G protein coupled receptors, and transcription factors; this downregulation is observed in both parasitic and free-living nematodes. Later, at the onset of heavy blood feeding, C-lectin genes were upregulated along with genes for Secreted Clade V Proteins (SCVPs), encoding a previously undescribed protein family that is conserved throughout clade V nematodes, but that is greatly expanded in the genomes of the blood-feeding parasites *A. ceylanicum*, *N. americanus*, and *H. contortus*. In addition, some of the upregulated C-lectin genes encode products that resemble vertebrate more than nematode lectins. Thus, the *A. ceylanicum* genome encodes large quantities of genes encoding stage-specific, secreted proteins that could serve as immunological decoys. At the same time, it encodes a much smaller set of potential drug or vaccine targets. Key drug targets consist of genes whose products are likely to be vital for function (as determined both by wide conservation in other parasites and by deleterious RNAi phenotypes in *C. elegans*) and yet are missing from mammals; chemical inhibition of their products should kill hookworms without harming the host. Key vaccine targets consist of genes whose products are likely to be immunologically accessible and crucial for survival: either proteases or protease inhibitors that were predicted to be secreted, were permanently upregulated after infection, and that lacked mammalian orthologs, but had *H. contortus* homologs that are also upregulated during infection. These criteria identified five proteases and one protease inhibitor gene whose transcripts comprised ~1% and ~0.1% of all protein-coding transcripts by adulthood. These findings should help elucidate pathogenesis by both hookworms and other strongylid parasites.

How we used *C. elegans* and hookworms to optimize an anthlemintic

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The soil-transmitted helminths or nematodes (hookworms, whipworms, and Ascaris) are roundworms that infect more than 1 billion of the poorest peoples and are leading causes of morbidity worldwide. Few anthelmintics are available for treatment, and only one is commonly used in mass drug administrations. New anthelmintics are urgently needed and crystal (Cry) proteins, in particular Cry5B, made by *Bacillus thuringiensis (Bt)* are promising new candidates. Cry5B has anthelmintic against many free-living and parasitic nematodes, including *in vivo* against multiple parasitic infections in rodents (*Heligomasmidoes polygyrus* and *Ancylostoma ceylanicum*) and in pigs (*Ascaris suum*).

Our group routinely works between *Caenorhabditis elegans* and intestinal parasitic nematodes such as the hookworm *Ancylostoma ceyalnicum*. Here we describe our work to optimize Cry5B sequence for anthelmintic activity. *C. elegans* was used for a large throughput-screen to select for Cry5B sequences with better anti-nematode activity. Various life-stages of hookworms were then used to validate and refine the hyper-activity mutants that were ultimately tested *in vivo*. Here, we will discuss how we work between these various nematode systems.

A microfluidic screening platform using electrophysiological recordings from parasitic larval stages of hookworm (*Ancylostoma ceylanicum)* and roundworm (*Ascaris suum).*

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New anthelmintic drugs for human and animal use are urgently needed. Several screening methods are available to help identify promising compounds, typically using worm motility as the readout. We previously validated in the free-living nematode, *Caenorhabditis elegans*, a microfluidic device ('chip') that records the electrophysiological signals generated by rhythmic contraction (pumping) of the worm's pharynx. These recordings, called electropharyngeograms (EPGs), are obtained from 8 worms simultaneously and provide an automated, medium-throughput quantification of muscular and neural activity at millisecond resolution. *C. elegans* is useful for high-content screening and molecular-genetic approaches but has significant physiological differences from parasitic nematodes. The current study tested the suitability of *Ancylostoma ceylanicum* (hookworm) and *Ascaris suum* (roundworm) nematodes for use with the EPG platform; *A. ceylanicum* is a human parasite and *A. suum* is a zoonotic model for the human parasite, *A. lumbricoides*. Microchannel dimensions in the EPG chips were customized for these species and chips were maintained near mammalian body temperature during recordings. Fourth-stage larvae (L4s) of *A. ceylanicum* removed from the intestine of hamster hosts exhibited robust, persistent EPG activity suitable for compound screening. This activity did not require the presence of serotonin (5-hydroxytryptamine; 5HT). The anthelmintic drug ivermectin (IVM) inhibited EPG activity, as did aqueous extracts of bitter melon leaves, *Momordica charantia*, a traditional anthelmintic treatment used in Haiti. We also recorded robust EPG activity in *A. suum* L3s removed from pig lung, in the presence of 5HT. This activity was inhibited by IVM. Our experiments validate the use of *A. ceylanicum* L4s and *A. suum* L3s with the microfluidic EPG platform, providing an important new tool for anthelmintic drug screening or studies of parasitic nematode feeding behavior. Microfluidic EPG chips for parasitic nematodes are available at NemaMetrix Inc. (nemametrix.com). Support: Bill & Melinda Gates Foundation, Grand Challenges Explorations award to JCW.

"Bridging the Divide" abstract: High Throughput Chemical Genomics in *C. elegans and P. pacificus*: Applications for discovering new anthelmintics and their targets

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More than 1.5 billion people, or 24% of the world's population, are infected with soil-transmitted helminth infections worldwide. These parasites cause a broad range of debilitating medical conditions primarily in the developing nations; chronic infections also cause severe malnutrition, which can lead to serious cognitive impairment. Only a few classes of anthelmintic drugs are available and resistant strains are emerging leading to treatment failure. Development of new anthelmintics is needed, however, the complex life cycle of parasitic helminths which relies on a propagation in a host animal makes it difficult to identify new compounds in high-throughput. The non-parasitic nematode *Caenorhabditis elegans* is amenable to high-throughput screening and molecular genetic techniques and could offer a powerful platform for anthelmintics discovery and mode of action studies.

We designed and built a fully automated High-throughput screening (HTS) robotic platform for chemical and functional genomic screening suitable for both whole-organism and cell-based assays. We are using *C. elegans* and its distantly related species *Pristionchus pacificus* as models for parasitic nematodes. With a platform capacity of ~2000 microplates and a life cycle of 3-5 days for our animal models, we can investigate up to 45,000 chemical compounds weekly and two genome-wide RNAi screens monthly. Workflows were optimized and our screening strategy was validated using an FDA approved library of ~2,000 compounds (Spectrum Collection, MicroSource). Known anthelmintic compounds were identified in the screen and caused paralysis or lethality in *C. elegans* and/or *P. pacificus*.

We are currently screening a custom pilot library of ~32,000 molecules (Chembridge) designed using a computational learning approach to predict internalization and bioavailability in the worm. Modifiers of chemically induced phenotypes, i.e. enhancer or suppressor genes will be identified using combinatorial assays of bioactives with RNAi or forward mutagenesis which will inform us about putative pathways and specific molecular targets. Toxicity of identified potential candidates will be assayed in mammalian cells using our HTS platform. We will present our preliminary results and future plans to identify new bioactive compounds with potential anthelmintic activity.

Bridging the Divide Workshop, 20th International *C. elegans* meeting.

The role of amphid signalling in ivermectin-mediated inhibition of reproduction in parasitic nematodes.

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The macrocyclic lactone drug ivermectin has revolutionised the control of river blindness, a disease caused by infection with the filarial nematode *Onchocerca volvulus*, and of other filarial infections such as lymphatic filiariasis. The pathology of river blindness is caused by the host immune response to the presence of L1 stage parasite larvae (microfilaria) in the skin and eyes. Ivermectin arrests the progression of this pathology and relieves acute symptoms by first removing microfilaria from the skin and second acting as a contraceptive of adult female parasites so that up to one year is required before new microfilaria re-populate the skin. The combination of these activities has led to the near elimination of river blindness from central and south America and from some previously hyperendemic regions of sub-Saharan Africa. At a molecular level, ivermectin is an irreversible agonist of inhibitory glutamate gated chloride channels on neurones and at the neuromuscular junction but the link between this molecular mode of action and the observation of potent, sustained inhibition of female reproduction by ivermectin is unclear. We have used the ivermectin induced inhibition of egg production in *C. elegans*, and the analysis of ivermectin resistant mutations, to show that (a) ivrmectin is a potent inhibitor of chemoreception at sub-nanomolar concentrations in *C. elegans*, (b) that signalling from the amphid neurones is a critical requirement for ivermectin inhibition of growth and egg production and (c) that ivermectin inhibition of feeding and egg production can be rescued by serotonin and drugs that increase serotonin levels (eg tricyclic antidepressants). These observations have led us to the hypothesis that amphid signalling determines ivermectin sensitivity by modulation of the balance between serotonin and glutamate's neurohormonal action in determining the excitability of motor neurones and muscles required for feeding (pharyngeal pumping) and egg laying (HSN's and uterine muscles). This is consistent with the observation that genes required for neuronal signalling are enriched strongly in regions of the genome of *O. volvuls* under selection in parasite populations with altered sensitivity to ivermecin, and demonstrates the utility of *C. elegans* as a model for studies of anthelmintic function.

IWM 2015 Abstract

Using bacterial pathogens to explore the nematode surface coat

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The surface of nematodes is poorly characterized, despite its critical roles in structural integrity, locomotion and protection from environmental stresses, as well in constituting a major interface with the mammalian immune system in parasitic nematodes. We have used bacterial pathogens that naturally adhere to the surface of *C.elegans* to identify mutants with altered surface biochemistry. We have thereby identified many genes involved in surface coat synthesis, which encode a variety of proteins implicated in glycosylation (glycosyl transferases and sugar transporters) and GPI anchorage. Mutant worms resistant to a virulent infection by one pathogen, Leucobacter Verde2, exhibit a trade off in being hypersensitive to the related "worm-star" pathogen Leucobacter Verde 1. Selections for resistance to Verde1 reveal new mutant classes. Many mutants affect the gene AGMO-1 (alklyglycerol monooxygenase), or enzymes required for the synthesis of its co-factor tetrahydrobiopterin. AGMO-1 is uniquely required for the breakdown of ether lipids, suggesting that these unusual lipids play an important role in the worm surface. To complement these genetic approaches we are characterizing surface-related lipids and proteins, in both *C. elegans* and parasitic nematodes.

Pilot studies to determine if dietary polyunsaturated fatty acids cause sterility in plant-parasitic nematodes

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Plant parasitic nematodes are one of the major pests affecting agricultural crops. Root-knot nematodes (RKNs) are among the most damaging group of plant parasitic nematodes. These nematodes are obligate parasites that infect their hosts in the root system. These parasites lead to a loss in excess of $100 billion for farmers due to reduced crop yield. Currently, the means of control for plant parasitic nematodes are chemical pesticides and resistant cultivars. However, many of the chemicals have been banned due to health concerns and certain populations of the nematodes have been able to overcome the resistance in selectively bred cultivars. As a result, a new method of control needs to be developed. We previously showed that when *Caenorhabditis elegans* are cultured on media containing specific polyunsaturated fatty acids (PUFAs) they become sterile. This led us to hypothesize that if plant parasitic nematodes are exposed to these particular PUFAs, they will display a reduction in the production of offspring. We obtained transgenic Arabidopsis plants expressing a specific PUFA and are currently conducting experiments to determine if this PUFA can reduce infection rates and reduce fertility of the root knot nematode *Meloidogyne incognita*. This represents a novel technique for controlling plant parasitic nematodes.

***Meloidogyne hapla*: a robust model for genetic and structural analyses of parasitism.**

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Growing sufficient food to meet the demands of an expanding human population cannot be achieved without increasing the productivity of existing farmland. As root-knot nematodes (RKN; *Meloidogyne* spp.) reduce global yield by approximately 10%, their control would have an immediate and profound impact on human wellbeing. In some instances, *C. elegans* can be used to model parasites. For example, we identified the *M. hapla* orthologue/analogue of *daf-12* and *daf-9* and found both to be constitutively expressed in the infective L2 (the dauer stage for RKN). Thus the L2 is primed for recovery, presumably only needing a sterol substrate (obtained upon feeding) to exit dauer. Using mass spectrometry, we discovered that in *M. hapla*, the vitellogenins accumulate in the L2. This heterochronic switch (compared to *C. elegans*) likely reflects the lack of a gut in the adult RKN. Like other obligate parasites, achieving reproductive status requires RKN to execute complex developmental and behavioral routines that require contributions from many genes. Consequently, and in contrast with *C. elegans*, most RKN genes are "essential". So, rather than perform *in vitro* mutagenesis, we have exploited the natural variation present within field populations. Linkage maps independently constructed from crosses of different strains exhibit robust concordance, and each is anchored to a full genome sequence. Using the maps, we have identified Mendelian loci conditioning parasite-related biology, and cloned candidate genes. But much beyond a platform for comparative analysis, the maps permit direct analysis of parasitic phenotypes. In particular, we asked: "which worm loci influence expression of which host loci." Using a cross-species, eQTL analysis we identified specific plant genes, many of which are controlled by a single RKN locus (on LG 3). One broad QTL influencing egg number revealed a family of RKN genes with coding potential for 12 members of the CEP family of plant peptide hormones. Bioassay showed that during parasitism, RKN encoded CEP phenocopy indigenous hormones; we propose the name "xenomone" for worm replicas of plant hormones. Comparison of tertiary structures of plant and nematode encoded CEP hormones (solved by NMR) revealed family-specific structural properties that have been implicated in receptor binding. Molecular dynamic simulations demonstrated relative conformational plasticity of CEP, especially in the amino and carboxyl arms; this is consistent with the broad host range of RKN. These analyses implicate xenomones as core communicative signals in the plant-nematode interaction, and as potential targets for novel anthelminthics. Geographical areas where subsistence agriculture predominates often have a high prevalence of human pathogenic helminths, exacerbating malnutrition. We suspect that management plans for helminth control might benefit from consideration of agricultural practices, and vice versa.

A Platform for Anthelmintic Drug Discovery using Genome-modified C. elegans

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Drug resistance is now occurring to all anthelmintics at market, yet drug development is mainly focused on introducing new molecules to the existing targets. As a result, drug resistance is quickly jumping to the new drugs and they become ineffective shortly after market introduction. Drug development needs to refocus on finding drugs with activity on alternative targets. The daf-12 signaling pathway is a promising new target for drug development, which may contribute in part to the complex therapeutic effects of ivermectin. The natural ligand of the daf-12 nuclear hormone receptor is dafachronic acid, which promotes entry into vegetative growth in both nematodes and parasites. Agonist and antagonists of daf-12 hold promise to be a novel class of therapeutics for controlling parasite infections in livestock and companion animals. AxumBio has developed a high-throughput approach for discovery of daf-12 therapeutics. A platform for detecting parasite-specific activity is created in *C. elegans* by inserting a parasite ligand binding domain (LBD) in place of the native LBD for daf-12. Multiple biomarkers of daf-12 signalling activity have been confirmed by qPCR. Using patented technology, biomarkers are converted to fluorescent reporters of gene activation to enable high-throughput plate reader assays. Ligand libraries are being screened at biomarker loci and ligands with nanomolar effectiveness are selected for screening in therapeutic use assays. By inserting parasite genes into the *C. elegans* genome, a high-throughput drug screen is created for detecting activity in a whole animal model. The drug screening platform is being adapted to a wide variety of parasites. The lead platform uses the *L. loa* LBD in replacement of the native daf-12 LBD (paraDAF12-Lloa). Activity data demonstrates the paraDAF12-Lloa chimera completely rescues dafachronic acid signalling function by promoting exit from daurer (parasitic larval) life stage. Two other platforms in development are *D. immitus* (paraDAF12-Dimm) and *T. Canis* (paraDAF-12-Tcan), which can be used to battle the growing incidence of drug resistance in companion animal therapeutics. Platforms to 20 parasites of various social and economic importance are also under development. Because the platform can be tailored to detect specific activity to a wide variety of parasites, the method provides a rapid path to discovery and eventual introduction of a novel class of anti-parasite drugs with both specific and broad-spectrum activity.