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A perspective on genomic-guided anthelmintic discovery and repurposing using *Haemonchus contortus*

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ABSTRACT

High-throughput molecular and computer technologies have become instrumental for systems biological explorations of parasites. Investigating the genomes and transcriptomes of different developmental stages of parasitic nematodes can provide insights into gene expression, regulation and function in the parasite, which is a significant step toward understanding their biology as well as host interactions and disease. This article covers aspects of a talk given at the MEEGID XII conference in Thailand in 2014. Here, we refer to recent studies of the genomes and transcriptomes of socioeconomically important parasitic nematodes of animals; provide an account of the barber's pole worm (*Haemonchus contortus*) and emerging drug resistance problems in this and related worms; we also propose a genomic-guided drug discovery and repurposing approach, involving the prediction of the druggable genome, prioritization of drug targets, screening of compound libraries against *H. contortus* and, briefly, a hit-to-lead optimization approach. We conclude by indicating prospects that molecular tool kits for nematodes provide to the scientific community for future comparative genomic, genetic, proteomic, metabolomic, evolutionary, biological, ecological and epidemiological investigations, and as a basis for biotechnological outcomes and translation.

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1. Introduction

Parasitic helminths (= worms) of animals cause diseases of major socio-economic importance globally (Anderson, 2000). In particular, parasitic roundworms (nematodes) have a considerable, long-term impact on animal health and cause suffering and economic losses of the tens of billions of the dollars annually (cf. Knox et al., 2011; Roeber et al., 2013). Historically, parasitic worms of animals have been studied to test drugs and vaccines, to investigate immune responses and to analyze pathogenic mechanisms in animal hosts. However, little is known about their fundamental molecular biology. Exploring the genomes, proteomes and metabolomes of worms should lead to essential knowledge and understanding of many key molecular events linked to parasite development and invasion of the animal host as well as disease processes and drug resistance. Clearly, this understanding, through the use of an integrated 'systems biology' approach, should have important implications for developing new ways of controlling parasitic worms through the disruption of one or more biological pathways. This focus is important, given the emergence of drug resistance in nematodes (Wolstenholme et al., 2012; Kaplan and Vidyashankar, 2012). Therefore, characterizing and comparing key nematode genomes and transcriptomes should assist in the development of innovative intervention strategies for parasitic diseases.

Over the years, progress has been made on characterizing transcriptomes and genomes of socioeconomically important animal-parasitic nematodes using Illumina-based sequencing technology and advanced bioinformatics. Draft genomes produced using this approach include those of the strongylid nematodes *Haemonchus contortus* (barber's pole worm; Laing et al., 2013; Schwarz et al., 2013), *Necator americanus*, *Ancylostoma ceylanicum* (hookworms; Schwarz et al., 2015; Tang et al., 2014) and *Angiostrongylus cantonensis* (rat lungworm; Yong et al., 2015); the ascaridoids *Ascaris suum* and *Toxocara canis* (ascaridoids; Jex et al., 2011; Zhu et al., 2015); the filarioids *Dirofilaria immitis* (heartworm; Godel et al., 2012) and *Loa loa* (eyeworm; Desjardin et al., 2013); and the enoplideans *Trichinella spiralis* (trichina; Mitreva et al., 2011) and *Trichuris suis* (whipworm; Jex et al., 2014). For some nematodes, particularly strongylids, the genome sequence of the free-living worm, *Caenorhabditis elegans* (100 Mb), and associated biological information (Harris et al., 2014) provide a solid platform for comparative molecular analyses. *C. elegans* has a rapid life cycle and is easy to maintain *in vitro*, allowing profound genetic and functional genomic investigations. Its karyotype (five pairs of autosomes and one pair of sex chromosomes) is consistent with a range of socio-economically important strongylid nematodes, and the genome contains ~20,000 genes. A number of these parasitic nematodes are relatively closely related to *C. elegans*, supported by comparisons with publicly available datasets for other nematodes. Therefore, the likelihood that a gene from a bursate nematode has an ortholog in *C. elegans* is relatively high (usually 50-70%; e.g., Cantacessi et al., 2012, 2015; Schwarz et al., 2013; Tang et al., 2014; Schwarz et al., 2015), with the exception of genes associated with particular host-parasite interactions and parasitism. Therefore, extrapolation from the biology of one of the best-studied metazoan organisms, *C. elegans*, is of significant benefit for studying the molecular biology of parasitic nematodes, particularly strongylids.

2. *H. contortus* and drug resistance in strongylid nematodes

Parasitic nematodes of the order Strongylida (Anderson, 2000) cause substantial morbidity and mortality in animals and people worldwide, and also major losses to the global food production annually (Hotez et al., 2008; Knox et al., 2011; Roeber et al., 2013). For example, *H. contortus* is one of the most important nematodes of livestock worldwide, infecting hundreds of millions of small ruminants (including sheep and goats) and causing disease, deaths and economic losses estimated at billions of dollars per annum. This nematode feeds on blood in the stomach and causes gastritis, anaemia and associated complications (haemonchosis), leading to serious production losses and death in severely affected animals (Sutherland and Scott, 2010). *H. contortus* is transmitted orally from contaminated pasture to the host though a complex life cycle (Veglia, 1915): eggs are excreted in host faeces; the first-stage larva (L1) develops inside the egg to then hatch (within 1 day) and develop through to the second- and third-stage larval stages (L2s and L3s) in about a week; the infective L3s are then ingested by the host, exsheath (xL3) and, after a histotrophic phase, develop through fourth-stage larvae (L4s) to dioecious adults (within 3 weeks) in the stomach.

The control of *H. contortus* and related strongylids relies largely on the use of anthelmintic drugs such as benzimidazoles (e.g., albendazole), macrocyclic lactones (e.g., ivermectin and moxidectin) and amino-acetonitrile derivatives (e.g., monepantel) (Holden-Dye and Walker, 2014). However, anthelmintic resistance in these worms is widespread or emerging (e.g., Wolstenholme et al., 2004, 2012; Kaplan and Vidyashankar, 2012). Although a parasite-derived vaccine (Barbervax; http://barbervax.com.au/) has been developed against *H. contortus* and commercialized, to support treatment programs, its efficacy, when used routinely on a large scale in the field, is not yet known, such that the design of new drugs is considered pivotal to ensure sustained and effective control into the future. The development and commercialization of the compounds derquantel (Lee et al., 2001), emodepside (Harder and von Samson-Himmelstjerna, 2001) and monepantel (Kaminsky et al., 2008; Prichard and Geary, 2008) has provided hope for the development of new classes of nematocides, but success in discovering new drugs using conventional screening approaches has been relatively poor, and the identification of new intervention targets are limited due to a lack of a detailed understanding of the biology of parasitic nematodes, the pathogenesis of disease and parasite-host interactions at the molecular and biochemical levels. Investigations of the genomes and transcriptomes of *H. contortus* (see Laing et al., 2013; Schwarz et al., 2013) and related hookworms (Tang et al., 2014; Schwarz et al., 2015) might assist the design of new intervention strategies.

3. Genomic-guided drug discovery and repurposing

The global market for anti-parasitic drugs of animals and humans appears to be worth tens of billions of dollars each year (e.g., Martin and Robertson, 2010). Traditionally, the search for novel drugs against parasitic nematodes has been carried out using conventional methods (e.g., the inhibition of parasite growth and/or development *in vitro*). Genomic, proteomic and bioinformatic technologies can likely assist the search for new drugs based on an understanding of the molecular biology of parasites themselves. For the first time, such technologies are providing the tools and information needed for targeted, post genomic drug discovery. This was not the case some years ago, and the goal now is the identification of new candidate drug targets. However, the major challenge is not only to identify targets, but also to prioritize and evaluate them, such that available resources can be focused sharply on those most likely to lead to effective treatments. The length of time and the prohibitive costs (estimated at $2.6 billion) linked to bringing a new drug to market (http://csdd.tufts.edu/reports/outlook_reports), together with the knowledge that most lead-compounds fail at some stage in the development process, have often deterred the pharmaceutical industry from investing in programs with little immediate financial return, such that, in our opinion, effective public-private partnerships are needed to make progress in this area.

4. The druggable genome, and prioritization of drug targets

The characterization of the draft genomes and developmentally staged transcriptomes of *H. contortus* elucidated, for the first time, the molecular biology of this parasite on a global scale (Laing et al., 2013; Schwarz et al., 2013). These genomes and transcriptomes now provide an important resource to the scientific community for a wide range of genomic, genetic, evolutionary, biological, ecological and epidemiological investigations, and likely a foundation for developing new interventions against *H. contortus* and possibly related strongylid nematodes. Here, we propose an integrated approach for the discovery and development of new drug targets and anthelmintic drugs (Fig. 1). A post-genomic pipeline should identify new drug target candidates, taking advantage of nematode and mammalian (host) sequence information available in current gene databases and incorporating gene function data for the free-living nematode *C. elegans* and/or other model organisms to produce a prioritized list of ‘nematode’ genes/gene products whose interruption or disruption adversely affects nematode survival and/or development (cf. Jex et al., 2011; Schwarz et al., 2013; Zhu et al., 2015).

Post-genomic drug target and drug discovery provides an adjunct to conventional screening and re-purposing (e.g., Loging et al., 2007; Woods et al., 2007; Agüero et al., 2008; Shanmugam et al., 2012; Taylor et al., 2013). The goal is to identify genes or molecules whose inactivation by one or more drugs will selectively kill parasites but not harm their host(s). As most parasitic worms, including *H.*

contortus, are difficult to produce and maintain without their host animal and functional genomic studies have been very challenging (reviewed by Geldhof et al., 2007; Knox et al., 2007; Lok, 2012), gene essentiality can be inferred from non-wild-type knock-down information (e.g., lethality) in *C. elegans* and/or other model organisms (Zhong and Sternberg, 2006; Lee et al., 2008). Using extensive genomic and transcriptomic resources now available for *H. contortus* (see Liang et al., 2013; Schwarz et al., 2013), druggable molecules can be predicted based on essentiality predictions, ensuring gene transcription in the parasitic stages of the parasite.

We have employed such an approach for proof-of-principle studies, and identified effective targets for nematocides (e.g., Campbell et al., 2011; Gordon et al., 2014). We have also suggested the use of a complementary approach to infer enzymatic chokepoints intrinsic to the metabolome of a parasite (Jex et al., 2011; Young et al., 2012). Such chokepoints are defined as enzymatic reactions that uniquely produce and/or consume a molecular compound; the disruption of such enzymes should lead to a toxic build-up (i.e., for unique substrates) or starvation (i.e., for unique products) of metabolites within cells (Yeh et al., 2004; Berriman et al., 2009). Chokepoints associated with essential molecules encoded by single-copy genes will have the highest priority. Using this strategy, we have predicted and prioritised drug targets in *H. contortus* (see Schwarz et al., 2013), which, given their relative conservation in invertebrates, are likely be relevant in relation to many other parasitic worms. To date, 260 druggable proteins have been predicted in *H. contortus*, of which 106 have ligands fulfilling the Lipinsky rule-of-five (Lipinksi, 2004) (Supplementary Table 1; Schwarz et al., 2013). Now, small molecule ligands that fulfil this rule can be prioritized, with a focus on channels and transporters, which represent protein classes known to be targets for anthelmintics, including macrocyclic lactones, levamisoles and amino-acetonitrile derivatives (cf. Campbell et al., 1983; Kaminsky et al., 2008; Qian et al, 2008) as well as on groups such as kinases, GTPases, GPCRs and/or phosphatases known to be specific targets for norcantharidin analogues (cf. Campbell et al., 2011). These prioritized ligands should then be the focus of future assessments.

5. Screening of compound libraries

H. contortus, which is relatively closely related to a wide range of socio-economically important strongylid nematodes and *C. elegans*, is a well-suited representative for drug discovery. This nematode is easy to maintain in small ruminants (sheep) in the laboratory, and has a short life cycle (3 weeks) compared with many metazoan parasites. Therefore, we recently established a whole-organism drug-screening assay to test prioritized chemical compounds on *H. contortus* (Fig. 2; Preston et al., 2015), and have shown that this method is readily applicable to other strongylid nematodes (Preston et al., unpublished data). The performance of this technique, which records larval motility microscopically by video-capture, has been rigorously evaluated and, recently, image capture, compound and worm dispensing into screening plates have been automated. This assay achieves high levels of repeatability and low levels of intra- and inter-assay variabilities (Preston et al., 2015), and has significant advantages over conventional methods (such as larval development, motility and migration inhibition methods; cf. Le Jambre, 1976; Martin and Le Jambre, 1979; Dobson et al., 1986; Kotze et al., 2006; Demeler et al., 2010), particularly in terms of ease of use, accuracy of results, throughput, time and cost, and compares very favourably with other assays developed for parasitic worms (e.g., Smout et al., 2010; Marcellino et al., 2012; Paveley et al., 2012; Paveley and Bickle, 2013; Hurst et al., 2014; Storey et al., 2014).

In our assay, chemicals (10 µM or 20 µM) are initially screened on exsheathed third-stage larvae (i.e. the first parasitic stage) of *H. contortus* in 96-well microculture plates using relevant controls (compounds: moxidectin and monepantel) (Preston et al., 2015). Following the recording of motility of xL3s at 48 or 72 h, the larvae are incubated for four more days to assess their ability to reduce development to L4s (cf. Sommerville, 1966). Data analyses are conducted, such that raw data are normalised against motility of the positive and negative controls (to remove plate-to-plate variation) by calculating the percentage of motility using the program Prism (v.6 GraphPad Software, USA). Z'-scores are calculated to validate the performance of the screening assay; reliable assays achieve Z'-scores of between 0.5 and 1 (Zhang et al., 1999). A compound is recorded as having activity if it reduces xL3 motility by $\geq$ 70% at 72 h. Compounds with anti-xL3 activity are screened three times at 10 or 20 µM to verify the repeatability of inhibition. For such compounds, dose-response curves over

time (usually 24 h, 48 h and 72 h) are produced for larval motility and L4 development to establish IC_{50} values; to do this, compound concentration is log10-transformed, and a variable slope four-parameter equation used. For compounds shown to repeatedly inhibit xL3 motility by ≥ 70%, the effect of the compound on the motility and growth of L4 (haematophagous, parasitic stage) is also assessed.

To date, in a public-private partnership with GlaxoSmithKline, we have screened a panel of 522 well-curated kinase inhibitors (GSK; code: PKIS2) for activity against *H. contortus*. We identified two chemicals (codes: GW800172X and GW461487A) within the compound classes biphenyl amides and pyrazolo[1,5-α]pyridines (cf. Angell et al., 2008a-d; Cheung et al., 2008), which reproducibly inhibit both xL3 and L4 motility and development, with IC_{50} values of 14-47 µM (Preston et al., 2015). Since these inhibitors were designed as anti-inflammatory drugs for use in humans, and fit the Lipinski rule-of-five (including bioavailability), they show considerable promise for hit-to-lead optimization and repurposing for use against various parasitic nematodes. Global transcriptomic and proteomic analyses of *H. contortus* (treated *versus* untreated), employing available molecular data sets (Schwarz et al., 2013) in a comparative manner, should inform about the pathways in the parasite being targeted by the chemicals. This approach might also be able to inform chemistry during target optimization.

6. Proposed hit-to-lead phase

We envisage that subsets of prioritized compounds, inferred to cause severe (lethal or semi-lethal) phenotypes in *H. contortus*, will be assayed in the short survival period following treatment. To do this, we plan to use a combined approach of advanced, high-resolution microscopic imaging (Preston et al., 2015) and metabolomics. Metabolomics should provide an effective way of establishing which biochemical pathways in *H. contortus* are affected by individual compounds, and will be highly complementary to transcriptomic and proteomic analyses (reviewed by Mikami et al 2012; Vincent and Barrett 2015). Our goal is to study individual phenotypes of various developmental stages of the parasites during treatment with particular drug candidates (different dosages and time points), and quantitatively assess their metabolic profiles compared with untreated controls, which will enable us to verify that chemical knock-down relates specifically to particular pathways or metabolic chokepoints inferred from the druggable genome (cf. Schwarz et al., 2013). Then, metabolic effectors could be studied *in vitro* using microsomes (phase I drug metabolism reactions) and hepatocytes (both phase I and II drug metabolism reactions) (e.g. Jia and Liu 2007; Vokral et al., 2013; Stuchlikova et al., 2014).

We propose to identify structural analogues of initial hit-compounds by similarity, substructure and pharmacophore searches of curated, open-access and in-house databases and synthesized. Based on this first round of screening (and depending on the number of fundamentally different chemical scaffolds identified as effectors), initial structure-activity relationships (SARs) could be established to inform the prioritization of particular chemotypes or scaffolds (reviewed by Guido et al., 2010; Hughes et al., 2011). For these selected scaffolds, focused libraries each comprising a series of compounds (based on a particular scaffold with various decorations) might be synthesized and then tested in our whole-organism screen (Preston et al., 2015). Informed by the outcomes from this screening round, the series of analogues will be tested in established assays to predict intestinal *a*bsorption, *d*istribution, *m*etabolism, *e*xcretion and *t*oxcitiy (ADMET) parameters (Jia and Liu, 2007). This step is critical, as many test compounds fail in development due to unforeseen, adverse side effects and/or poor pharmacokinetic profiles. In parallel, solubility and lipophilicity predictions will be made using established chemoinformatic procedures. Compounds with favourable descriptors and inferred to be metabolically stable (based on microsome testing) and safe (mammalian cells) would then be tested in sheep by oral, intravenous and percutaneous administration. *In vivo* plasma concentration profiles will be established by liquid chromatography mass spectrometry (LC-MS) to determine the metabolites involved in the elimination of the compound (Liu and Jia 2007). Any limitations, in terms of bioavailability, metabolism, toxicity and/or pharmacokinetics (e.g., poor solubility or short half-life), should be identified and addressed. At this point, a decision would be made as to whether an early lead compound is worthy of optimization. Candidate compounds selected to proceed on the basis of their effect on *H. contortus*, ADMET parameters and favourable pharmacokinetics would be re-synthesized, and then tested in sheep for their safety and ability to eliminate *H. contortus* infection. Organs and tissues (including liver, spleen and central nervous

system) would be examined for macro- and histo-pathological changes. Subsequently, the optimum dosage, and frequency and route(s) of administration as well as formulation of chemicals would be established.

7. Concluding remarks

The draft genomes and transcriptomes of selected strongylid nematodes, including *H. contortus*, *N. americanus*, *Anc. ceylanicum* and *Ang. cantonensis*, provide useful molecular tool kits to the scientific community for many comparative genomic, genetic, proteomic, metabolomic, evolutionary, biological, ecological, and epidemiological investigations. Importantly, the integrated use of advanced genomic, transcriptomic, proteomic and bioinformatic methods will likely support investigations of the structures and functions of key molecules essential to the development, reproduction and survival of parasitic nematodes, and should pave the way for the prediction, prioritization and/or design of small molecular inhibitors as new nematocides. To this end, recently developed drug-screening assays that use the parasitic stages of worms and have medium-throughput (reviewed by Paveley and Bickle, 2013; Preston et al., 2015) should be adaptable to various parasites of socioeconomic importance, including those causing neglected tropical diseases. We consider this focus to be of considerable relevance, given the need to meet the goals of the London Declaration (http://unitingtocombatntds.org/resource/london-declaration). Our goal would be to contribute through rapid and efficient repurposing of compounds in public-private partnerships.

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Supplementary Table 1. Drug target candidates predicted for *Haemonchus contortus* that have ligands that pass the Lipinski's rule-of-five (cf. Schwarz et al., 2013).

Category	Class	Family
Transcription factors	Superclass: Basic Domains	Class: Helix-loop-helix factors (bHLH)
	Superclass: Helix-turn-helix	Class: Fork head / winged helix
		Class: Homeo domain
	Superclass: Zinc-coordinating DNA-binding domains	Class: Cys2His2 zinc finger domain
		Class: diverse Cys4 zinc fingers
RNAi machinery	SMRNA_BIOSYNTH	XPO-1
		DCR-1
Phosphatases	Fructose-1,6-bisphosphatase I [EC:3.1.3.11]	Other
	Protein phosphatase 2 (formerly 2A), regulatory subunit B	2A
	Uridine phosphorylase [EC:2.4.2.3]	Other
Kinases	AGC	PKN
		RSK
	Arginine kinase [EC:2.7.3.3]	arginine kinase [EC:2.7.3.3]
	CAMK	MAPKAPK
		PIM
		RAD53
		MAPKAPK
		MLCK
	CK1	CK1
	CMGC	CDK
		MAPK
	Others	Aur
		RIO
	STE	STE20
	TKL	MLK
		RAF
	Tyrosine protein kinases	Csk
		FGFR
		InsR
GTPases	Small GTPase	Rho
GPCRs	Class A	A7
Channels	Electrochemical potential-driven transporters	2.A.55
		2.A.6
	Group translocators	4.C.1

		4.C.2
	Incompletely characterized transport systems	9.A.40
		9.B.45
		9.B.45
	Primary active transporters	3.A.1
		3.A.16
		3.A.18
		3.A.20
		3.A.3
Other	Aminotransferase	tyrosine aminotransferase [EC:2.6.1.5]
		aspartate aminotransferase, mitochondrial [EC:2.6.1.1]
	Cellular antigens	peptidyl-dipeptidase A [EC:3.4.15.1]
		disintegrin and metalloproteinase domain-containing protein 17 [EC:3.4.24.86]
	Chaperones and folding catalysts	heat shock protein 90kDa beta
		protein disulfide isomerase family A, member 3 [EC:5.3.4.1]
	Cyclins	cyclin D2
	Cytoskeleton	kinesin family member 5 Cellular Processes
	Fatty acid synthase	3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]
	GTP-binding proteins	guanine nucleotide-binding protein G(i) subunit alpha
	Hydrolases	dCMP deaminase [EC:3.5.4.12]
		kynureninase [EC:3.7.1.3]
		carboxypeptidase A2 [EC:3.4.17.15]
		mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [EC:3.2.1.96]
		fatty acid amide hydrolase [EC:3.5.1.99]
	Ligases	carbamoyl-phosphate synthase / aspartate carbamoyltransferase [EC:6.3.5.5 2.1.3.2]
		carbamoyl-phosphate synthase large subunit [EC:6.3.5.5]
	Lyases	enoyl-CoA hydratase [EC:4.2.1.17]
		ATP citrate (pro-S)-lyase [EC:2.3.3.8]
		adenylate cyclase 9 [EC:4.6.1.1]
		adenylate cyclase 5 [EC:4.6.1.1]
		adenylate cyclase 2 [EC:4.6.1.1]

Oxidoreductases	malate dehydrogenase [EC:1.1.1.37]
	estradiol 17beta-dehydrogenase / testosterone 17beta-dehydrogenase / retinol dehydrogenase [EC:1.1.1.62 1.1.1.63 1.1.1.105]
	S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase [EC:1.1.1.284 1.1.1.1]
Replication and repair	exodeoxyribonuclease III [EC:3.1.11.2]
	NAD-dependent deacetylase sirtuin 1 [EC:3.5.1.-]
	cyclin A
	ribonucleoside-diphosphate reductase subunit M1 [EC:1.17.4.1]
	histone acetyltransferase [EC:2.3.1.48]
	DNA polymerase alpha subunit B
	histone deacetylase 3 [EC:3.5.1.98]
	DNA polymerase delta subunit 1 [EC:2.7.7.7]
	ATP-dependent DNA helicase RecQ [EC:3.6.4.12]
Spliceosome	polyglutamine-binding protein 1
Transferases	thymidylate synthase [EC:2.1.1.45]
	purine-nucleoside phosphorylase [EC:2.4.2.1]
	acetyl-CoA acyltransferase [EC:2.3.1.16]
	polypeptide N-acetylglucosaminyltransferase [EC:2.4.1.-]
	hydroxymethylglutaryl-CoA synthase [EC:2.3.3.10]
Translation	ribosome maturation protein SDO1
	translation initiation factor 2 subunit 1
	translation initiation factor 4G
Ubiquitin system	ubiquitin-like 1-activating enzyme E1 B [EC:6.3.2.19]
	ubiquitin-activating enzyme E1 [EC:6.3.2.19]

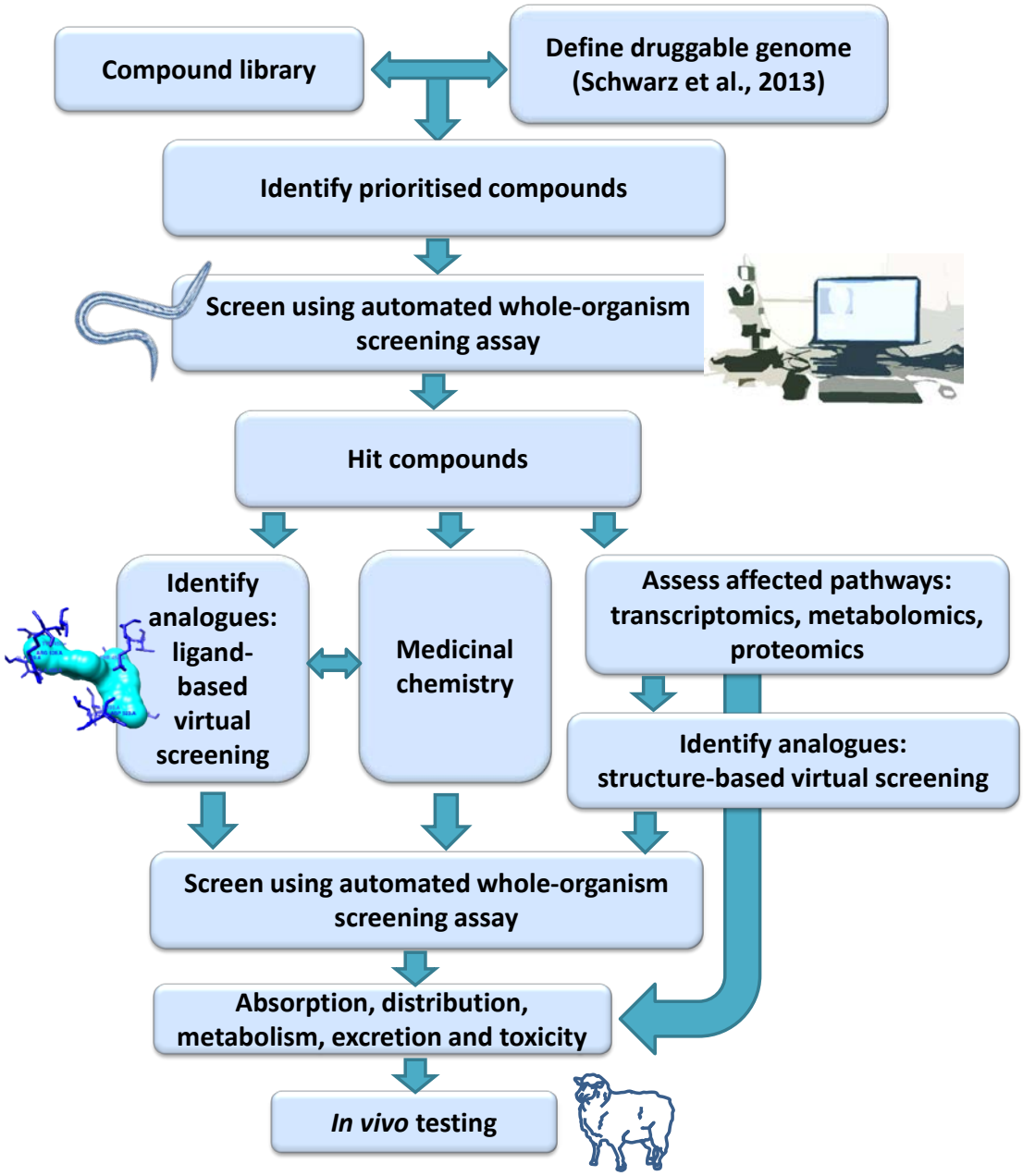

Fig. 1. Proposed integrated pipeline for the discovery and repurposing of drugs for parasitic nematodes using *Haemonchus contortus* as a model.

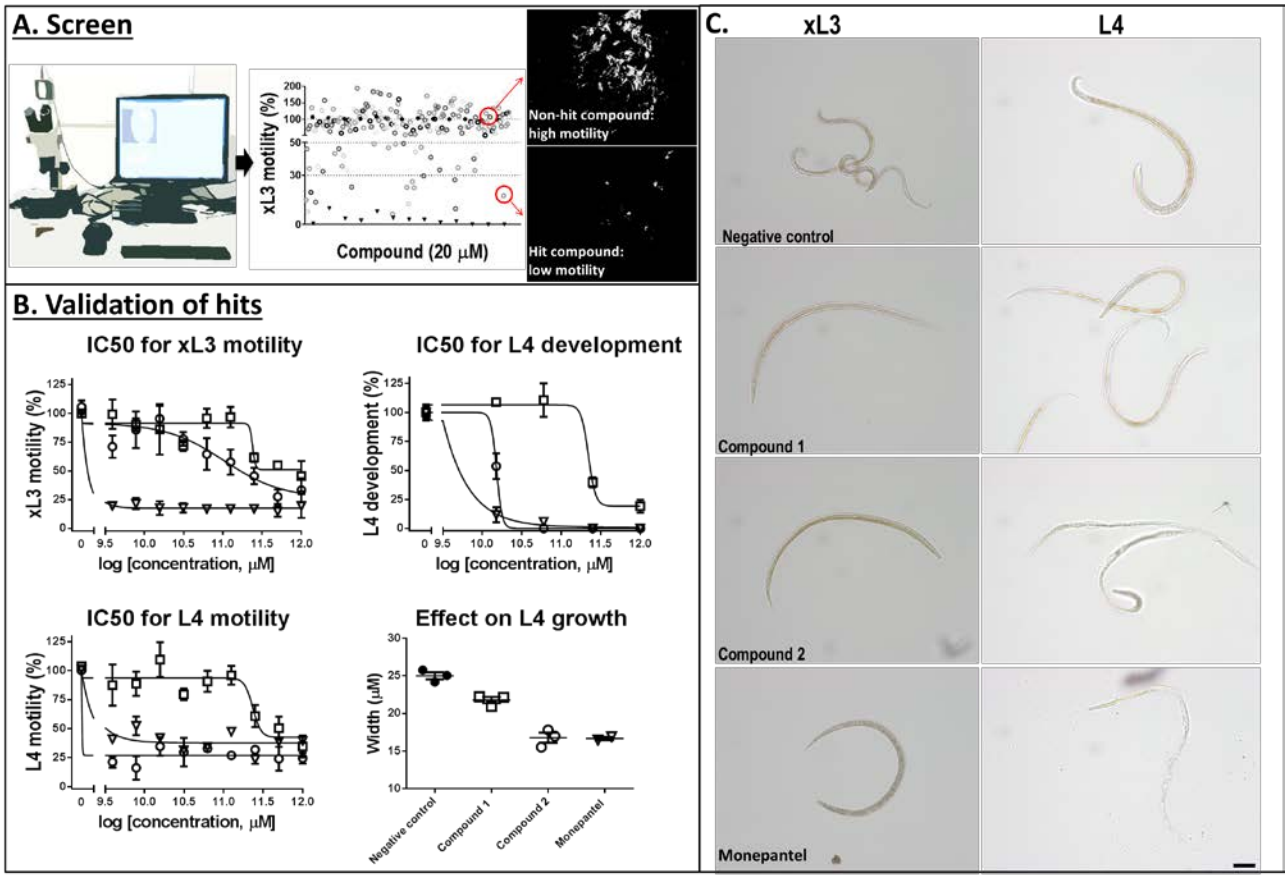

Fig. 2. Establishment and assessment of automated whole-organism drug screening assay (cf. Preston et al., 2015). A: Representative results from screen, showing worm larvae with high (blue, top right) and low (black, bottom right) motility after 72 h incubation with various compounds. **B:** New compounds and their effects on larval motility and development (dose response curves to establish half of the maximum inhibitory concentration; IC_{50}), and inhibition of larval growth. **C:** Phenotypic effects (light microscopy; scale bar = 50 µm) of hit compounds on third (xL3)/and fourth (L4) stage larvae (at 72 h).