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When Secretomes Meet Anthelmintics: Lessons for Therapeutic Interventions

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Helminth secretomes comprise many potential immunomodulators. The molecular and functional diversity of these entities and their importance at the host–parasite interface have been increasingly recognized. It is now common to hypothesize that parasite-derived molecules (PDMs) are essential mediators used by parasites to establish and remain in their hosts. Suppression of PDM release has been reported for two anthelmintic drug classes, the benzimidazoles and macrocyclic lactones, the mechanisms of action of which remain incompletely resolved. We propose that bringing together recent insights from different streams of parasitology research, for example, immunoparasitology and pharmacology, will stimulate the development of new ways to alter the host–parasite interface in the search for novel anthelmintic strategies.

Highlights

The molecular negotiations engaged between hosts and parasites have resulted in the evolution of highly specialized interspecific relationships in which an array of PDMs delimit conditions that render the host permissive for the parasite.

An underappreciated characteristic of some anthelmintics, such as ivermectin and albendazole, whose mechanisms of action remain incompletely resolved, is their ability to block the release of PDMs in culture.

Effects of these drugs on secretion correlate well with the spectrum of activity, kinetics of expulsion, and levels of drug exposure achieved *in vivo*, as shown experimentally for some parasitic nematodes.

Incorporating screens for the inhibition of PDM release in culture as a complement to existing drug discovery efforts may seed novel opportunities in the search for novel anthelmintic strategies.

Altering the Host–Parasite Interface as a Strategy for Helminth Control

Mammals, including humans, have been selected through evolution for resistance to almost all parasites. Nonetheless, certain species of parasites have evolved the ability to infect selected host species (permissive hosts) by overcoming or manipulating the normally highly effective host immune system. Understanding how parasite species colonize permissive hosts – while being excluded from nonpermissive hosts – has been a long-standing challenge in parasitology research, but recent advances in our ability to decipher the language of the host–parasite interface have begun to illuminate key factors that determine the outcome of this interaction [1]. The intriguing possibility of rationally converting permissive into nonpermissive hosts as a therapeutic strategy warrants more research into the composition of parasite **secretomes** (see Glossary), identification of the molecules that enable them to establish in the host, and the mechanisms by which these molecules are released to the host milieu.

Helminths are complex metazoan organisms that belong to different taxonomic families, many members of which have adopted a parasitic lifestyle. The pathologies they cause in humans and animals, and their deleterious impact on crop production, represent threats to socioeconomic development [2–5]. In addition, progression of drug resistance has been rapid, especially among livestock parasites [5,6]. Expansion of the toolkit for helminth control is urgently needed, as is the acquisition of a deeper understanding of the mechanisms behind current anthelmintic therapies, which might, in turn, inspire new approaches to therapeutic intervention. Focusing on nematodes, we present scenarios in which anthelmintic therapy seems to alter host–parasite crosstalk via the suppression of parasite excretion/secretion processes. We further propose that current anthelmintic screening approaches, from empirical to rational, should be expanded to incorporate these processes as an important source of drug targets. The literature suggests that these basic principles apply to helminths beyond the phylum Nematoda.

Helminth Secretomes: A Promising Source of New Drug Targets

The relationships between helminths and their hosts have been shaped by millions of years of coexistence and by an evolutionary arms race. Helminths have evolved various strategies to

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establish and remain in the inhospitable environment constituted by an engaged host immune response. From an immunological perspective, this interaction may be considered to have generally resulted in a state of 'disease tolerance' in the host associated with the presence of parasites [7], in a compromise between clearing worms and preventing extensive tissue damage due to the strong inflammatory immune response a large pathogen would normally elicit [8]. The molecular negotiations engaged between host and parasite have resulted in the evolution of highly specialized interspecific relationships in which secretomes [an array of parasite-derived molecules (PDMs)] delimit conditions that render the host permissive for the parasite, enabling a chronic infection [9]. In that context, the molecules released (e.g., proteins, microRNAs, metabolites) by parasites that modulate host immune responses are attracting increasing attention.

Historically, PDMs were identified through the analysis of helminth excretory/secretory (E/S) products, including in this context both excreted 'waste' products and actively released 'functional' molecules. Helminth E/S proteins have been the best studied PDMs in terms of host manipulation. Modern technologies then enabled in-depth characterization of additional PDMs, expanding the repertoire of potential immunomodulators beyond the protein compartment. These include nucleic acids, lipid-based bioactive molecules, carbohydrates (e.g., in the form of glycoproteins and glycolipids), and small metabolites [10–12]. The existence of miRNAs as PDMs has attracted increasing attention [13,14]. These small noncoding RNAs have important roles in regulating gene expression as they suppress translation of targeted mRNAs [15]. Horizontal transfer of miRNAs between parasite and host cells has been shown, and parasite miRNAs retain (at least partially) their regulatory functions in this context. Based on available data, it is hypothesized that miRNA release represents another widespread host-modulating approach used by helminths [16,17].

Some E/S products are encapsulated in extracellular vesicles (EVs) [18–20]. This is especially relevant for nucleic acids, which are quickly degraded outside the cellular milieu. Although our understanding of the mechanisms of EV-mediated communication remains incomplete, and is largely based on circumstantial evidence [21], roles for helminth-derived EVs in both regulation and promotion of inflammation have been reported (reviewed in [19,20]), following uptake by host cells.

Active PDM secretion typically occurs through specialized structures in nematodes, called excretory glands or secretory pore [22,23]. Other sources (e.g., cuticle) also contribute to the PDM composition [24] (Figure 1). EVs originate from intestinal cells and are released from the secretory pore, or from the nematode's anterior opening [25] and perhaps in uterine fluid [26]. It is generally accepted that helminth PDMs modulate host defense mechanisms and so represent essential mediators of parasitism [20,27,28]. Their presumed involvement in a range of processes likely evolves with the changing stress situations imposed by the parasite's life cycle in the host and requires continuous release of discrete PDM populations. PDMs display a wide variety of mechanisms of action, including mimicry of host molecules and neutralization of host effectors, among others [27,29]. The consequences for host cell functions are varied but critical: they may inhibit signaling cascades, protease activity, and many more key pathways, which overall favor infection [27]. The paramount importance of PDMs to sustain helminth infections is widely recognized, and hence has sparked interest in them as attractive targets for novel drug and vaccine discovery. Developments in this area require additional investment in research to improve the tools available to study parasite biology (Box 1) and to bolster our currently limited mechanistic understanding of PDMs and how they function in the host [25,30].

Identifying PDMs that make hosts permissive to infection should encourage efforts to rationally target their key components or the affected immune response pathways to restore the host's capacity to resist or eliminate the infection. Initially, discovery of new anthelmintics relied on

Glossary

Anthelmintic screening/screens: the process of testing the sensitivity of helminths to chemical compounds through *in vitro* or *in vivo* tests as a means to discover and develop efficacious drugs.

Benzimidazoles: an anthelmintic class to which drugs such as albendazole and mebendazole belong. The benzimidazoles represent one of the current mainstays in the control and treatment of human helminthiases. They act by inhibiting helminth microtubule polymerization.

Macrocyclic lactones: an anthelmintic class to which drugs such as ivermectin and moxidectin belong. This class is another main pillar sustaining the control and treatment of human and animal helminth infections. Macrocyclic lactones interfere with the helminth nervous system by preventing responses to excitatory neurotransmission.

Secretome: the complete set of parasite-derived molecules (PDMs) expressed by an organism and released by secretion into the environment. If the term usually refers to the protein fraction, we extend it here as the full spectrum of known and unknown molecules, including extracellular vesicles (EVs) and their contents, shed into the extraorganismal space by helminths.

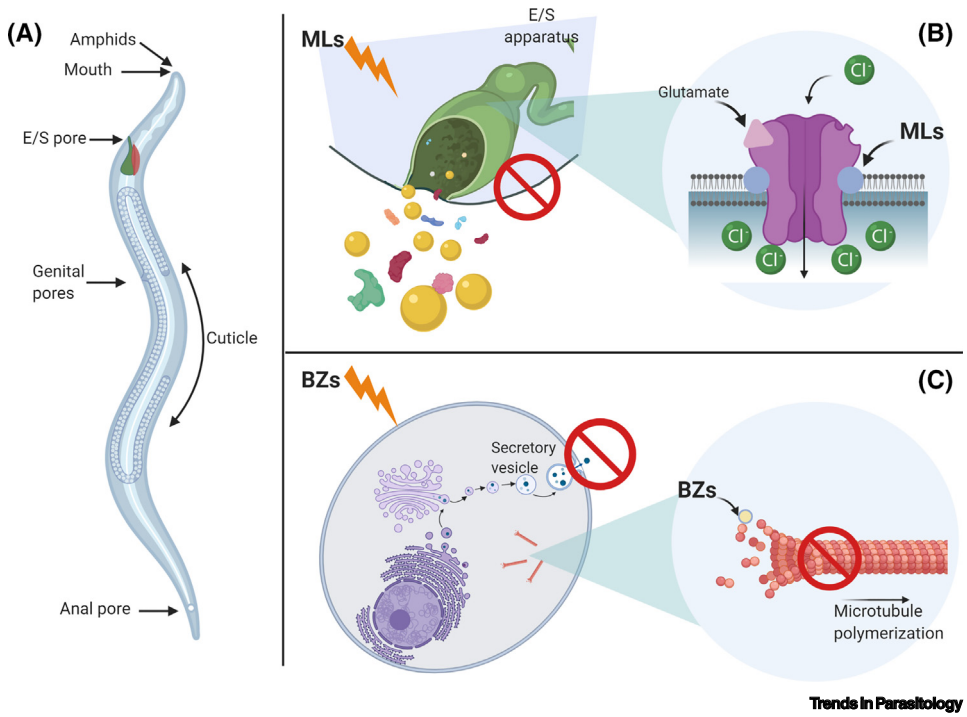

Figure 1. Mode of Action of the Macrocyclic Lactones (MLs) and Benzimidazoles (BZs). Anthelmintics affect various aspects of nematode physiology. We propose that the impairment of secretion of parasite-derived molecules (PDMs) may represent an additional and important aspect of their action on nematodes. (A) Multiple nematode structures are associated with PDM release. PDMs are proposed to be secreted or excreted via the excretory/secretory (E/S) pore as well as body openings [mouth, anus, genital pores, amphids (sense organs located on the head of nematodes, forming invaginations in the cuticle)], and from the cuticle. (B) The classic view of ML action at glutamate-gated chloride channels (GluCls), causing an increased cellular concentration of chloride ions, which leads to impaired muscle function and paralysis. In microfilariae, GluCls are associated with the E/S apparatus, indicative of neuromuscular regulation of its function. Exposure of microfilariae to MLs disrupts PDM release. (C) BZs inhibit tubulin polymerization, interfering with several cellular processes required for life. The absence of stable microtubules hinders the intracellular trafficking of PDMs to the plasma membrane and impairs secretion. The image was created with BioRender.com.

empiric observations of efficacy in infected hosts. This strategy evolved to the use of phenotypic **anthelmintic screens** (in which viability of parasites exposed to compounds is measured *ex vivo*), and to a lesser extent, on mechanism-based screens, which require recombinant expression systems to access selected targets [5,31]. These efforts are limited by the fact that the mechanisms of action of several anthelmintic classes remain incompletely resolved.

Box 1. Obstacles to Studying Helminth Parasites and Their Secretomes

As metazoan obligate parasites with complex and long life cycles, parasitic helminths are particularly challenging to study. The experimental availability of these parasites is facilitated by rodent models for some (but not all) species, but these are associated with high maintenance costs and ethical issues inherent to animal experimentation. It is currently not possible to maintain obligatorily parasitic helminths in culture for the whole life cycle, or under conditions that closely mimic the host. Consequently, helminths can be observed *in vitro* for only a few days, making direct comparison between specimens in culture and in *in vivo* settings inherently speculative and imperfect.

Methods to sensitively measure the secretion of PDMs are scarce, and a priori only very few components are common across helminth secretomes. In line with this, there is no consensus as to the identity of host functions affected by PDMs. Recent progress made with reverse genetics (CRISPR), however, enabled the production of a small proportion of *B. malayi* microfilariae that secrete luciferase [63]. This pioneer work may pave the way toward implementable tools for monitoring secretory activity both *in vitro* and *in vivo*. This will ultimately require that the genetic toolbox for parasitic species continues to improve, along with our abilities to edit parasite nuclear genomes in a heritable manner.

An underappreciated characteristic of some anthelmintics, such as ivermectin (IVM) and albendazole, is their ability to block the release of PDMs in culture [22,32–34]. Does this effect contribute to their mechanisms of action, which have focused on paralysis of somatic and pharyngeal muscles and disruption of the tubulin–microtubule equilibrium, respectively?

Do Anthelmintics Act by Disrupting Parasite Secretory Processes?

Beyond the recognition of helminth PDMs as key mediators of host–pathogen interactions, a limited amount of evidence is available from data generated *in vivo* to identify components that drive progression of infection and immune evasion, and that thus determine host–parasite specificity. Importantly, characterization of PDMs has led to the concept that therapies can be devised to disrupt the dialog between host and parasite. Work on vaccines that specifically target secreted antigens based on their known or presumed roles in enabling infection has shown that this approach can generate therapeutically useful outcomes. These efforts have also provided additional *in vivo* evidence for the roles of specific secretome components in infection [35–37].

Insufficient attention has been paid to the study and exploitation of parasite processes upstream of the release of biomolecules to the host. However, a closer look at the mode of action of anthelmintic **benzimidazoles** (**BZs**) and **macrocyclic lactones** (**MLs**) reveals aspects of their effects that support the relevance of PDMs in host–parasite interactions (Figure 1). In particular, they highlight aspects of parasitic nematodes that control the release of PDMs. Indeed, effects on secretion seem to correlate well with the spectrum of activity, kinetics of expulsion, and levels of drug exposure achieved *in vivo*, as shown experimentally for some stages of some parasitic nematodes. Whether inhibiting secretion is the key trigger for parasite clearance, or one of several detrimental pharmacological effects, remains to be determined.

BZs: The Role of Microtubules in Secretion

BZ anthelmintics act by inhibiting microtubule polymerization. Functional and genetic evidence supports the binding of BZs to β-tubulin as the basic mechanism of antiparasitic activity [38]. Key components of the eukaryotic cytoskeleton, microtubules have roles in cell division, cell shape, intracellular organization, transport, and the motility of cilia and flagella [39]. More recent evidence shows the involvement of microtubules in transport of cargo proteins from the Golgi to the cell surface and in exocytosis [40]. It is reasonable to infer that destabilization of microtubule assembly results in deep physiological alterations in parasitic nematodes, including disruption of the secretory pathway.

In vitro and *in vivo* observations describe different effects of BZs in larvae compared to adult stages. Development is arrested in embryonic and larval stages under transition at the time of BZ exposure [41,42]. These observations establish a link between an effect of BZs on the mitotic spindle and phenotypic consequences in terms of incomplete cell division. In contrast, BZ effects in adult stages seem to be cumulative over drug exposure and are characterized by damage to parasite external surfaces and intestinal tissues, which can impair nutrient uptake, transport, and energy metabolism [43]. Despite these effects, parasite motility, a major indicator of parasite viability, remains unaffected, at least acutely [42,44–47].

Disruption of parasite secretory processes seems to be the earliest physiological effect of BZ exposure on parasite stages for which a mechanistic explanation can be advanced based on a role for microtubules in secretory pathways [40]. Foundational work on ultrastructural changes in *Ascaris suum* and *Syngamus trachea* intestinal tissues following *in vivo* mebendazole exposure showed the accumulation of secretory granules as the earliest cellular effect accompanying the disappearance of microtubular structures [47]. Further observations, focusing on the secretion

of acetylcholinesterase (AChE) from *Nippostrongylus brasiliensis*, provide insight on the downstream effect of accumulation of these granules. While BZs induce accumulation of AChE in the organism, there is a rapid and marked suppression of AChE secreted to media [32,48]. Consistent results have been reported for AChE secretion by *Heligmosomoides polygyrus* adults [33] and total protein released by *Brugia malayi* microfilariae [22].

Thus, BZs inhibit protein secretion from parasitic nematodes. Whether this effect triggers parasite clearance remains unproven. However, an important aspect of the *N. brasiliensis* work is the correlation between the accumulation of AChE (due to inhibition of protein secretion) and the dynamics of worm expulsion *in vivo* [48], indicating that this is plausible, at least for gastrointestinal (GI) nematodes.

Rapid parasite expulsion is a hallmark of BZ therapy against GI nematodes [49–51], in contrast with the slower clearance/mortality seen in tissue parasites, which usually requires prolonged therapy [44]. Differences between tissue and GI parasite clearance/expulsion kinetics are partly the result of the duration of exposure to effective drug concentrations (pharmacodynamics), which is influenced by parasite biology, its host tissue location, and host immune competence. The reversible nature of the BZ–β-tubulin interaction might allow the reconstitution of the microtubule network and secretory function once local drug levels decrease, allowing tissue or circulating parasites to remain in the body and counteract immune responses mounted during the lapse in the shutdown of PDM release. Secondary effects resulting in cellular and surface damage might become primary clearance drivers when repeated/continuous drug exposure is needed to generate a therapeutic outcome.

The mechanisms leading to suppression of parasite development are not necessarily the same as those that trigger adult parasite clearance. Furthermore, although the physiological effects and cellular damage caused in worms by BZ exposure are relatively conserved, the mechanisms involved in clearance/destruction of tissue parasites must be different from those that drive efficacy against GI nematodes. For the latter, the pattern of exposure to BZs and the dynamics of parasite expulsion are compatible with a contribution of disrupted secretory processes as a cause of parasite elimination.

ML Mechanism of Action Reflects the Control of Secretory Processes by Neuromuscular Function

The spectrum of action and physiological effects of ML anthelmintics are explained by the location of glutamate-gated chloride channels (GluCls), their primary targets. GluCls are members of the large family of cys-loop ligand-gated channels; they are pentamers of one or more subunit types and respond to small neurotransmitter ligands [52]. MLs act as allosteric modulators of GluCls, resulting in pseudoirreversible hyperpolarization of postsynaptic nerve and muscle cells, preventing response to excitatory neurotransmission [53].

Physiological effects of exposure of nematodes to MLs reflect the anatomical distribution of GluCls. In GI nematodes, as well as in *Caenorhabditis elegans*, in which GluCls have been best characterized, MLs induce paralysis of somatic muscle-mediated locomotion and pharyngeal pumping and impair some sensory signaling pathways [54]. These effects can be explained by the localization of GluCls in cells that control these processes and suggest that GI parasite expulsion results from the disruption of one or more of them [55–57]. Less information is available for filarial nematodes despite the widespread use of MLs for chemotherapy of human and veterinary filariases. However, evidence is accumulating to support the conclusion that GluCls are involved in PDM release to the external environment via control of neuromuscular activity of

the secretory system. It is important to recognize that IVM also potently inhibits the release of PDMs (AChE) from GI nematodes [33], an effect that may contribute to their elimination from the host. More research is needed to resolve this situation.

ML activity against filarial nematodes *in vivo* leads most prominently to microfilaricidal and embryostatic activity. MLs also show potent activity against some but not all developing stages in some species, with outstanding efficacy against the heartworm *Dirofilaria immitis in vivo* [52,58]. The effects of ML exposure on motility and pharyngeal function described for GI nematodes have not been consistently reported in filariae at pharmacologically relevant concentrations, clearly distinguishing the mechanisms of anthelmintic efficacy between these groups of parasites.

In *B. malayi* microfilariae, GluCls are associated with a muscular structure surrounding the secretory vesicle, which is proposed to control release of PDMs [22]. *In vitro* exposure of microfilariae to IVM decreased the amount of protein released to culture media at concentrations as low as 100 nM [22]; this effect extends to the release of EVs from microfilariae, adults, and infective larvae [34]. Immunohistochemical analysis demonstrated that the E/S pore is the main source of protein release from *B. malayi* microfilariae [22] and possibly EVs [34], suggesting that release of PDMs into the host is a tightly regulated process. Although information on the localization of GluCls in other filarial parasite species remains incomplete, the observation of inhibitory effects in several stages of *B. malayi* indicates that PDM release is governed by neuromuscular processes distinguishable from those that control locomotion. In addition, these observations support the concept that microfilarial clearance from the host results from disruption of the molecular dialog between host and parasite that allows a chronic infection to be maintained.

Experimental evidence supporting a requirement for immune components for ML efficacy is available. For example, *in vitro* killing of *Acanthocheilonema viteae* and *Litomosoides carinii* microfilariae by IVM can be achieved in the presence of host immune cells and serum components [59,60]. More recently, IVM exposure during coculture of *D. immitis* microfilariae and canine neutrophils or peripheral blood mononuclear cells (PBMCs) has been shown to induce cell attachment to the worms at therapeutic drug concentrations [61]. These effects extend to moxidectin, another ML [62]. Remarkably, based on the concentration-dependence of the cell attachment response, it is possible to discriminate between different MLs based on potency in this assay system. In addition, the drug response in different parasite strains depends on the extent of resistance to MLs *in vivo* [62]. Assuming conservation across species in the localization of GluCls and their functional role in the microfilarial E/S apparatus, these results are consistent with ML-induced suppression of the release of PDMs as the cause of clearance of microfilariae from the host. Further work focusing on ML effects on PDM release from developing larvae across multiple filarial species will enable more detailed characterization of the host and parasite components involved in parasite clearance *in vivo*.

Concluding Remarks

Evidence is accumulating regarding the role of helminth PDMs as determinants of host specificity and survival. In particular, BZ and ML anthelmintics provide new insights on how PDM release is regulated. The pharmacology of these drugs highlights how interaction of PDMs with the host immune system contributes to parasite clearance. In this regard, our grasp of the repertoire of targets for anthelmintic chemotherapy or vaccination is incomplete (see Outstanding Questions). We need to develop integrated basic knowledge from different streams of parasitology research to expand our understanding of secretory processes, to characterize individual PDMs and to decipher their contribution to the sustained communication flow between parasite and host. Identification of key PDMs as determinants of the 'equilibrium' between host and parasite, as

Outstanding Questions

Is PDM release stimulated and/or modulated by host cues, or is the process constitutive and inherent to a given parasite stage?

Do PDMs have a systemic or rather local host modulatory potential?

To what extent does host–parasite specificity impose (technical) challenges on the search for new therapeutic targets?

Can we 'reduce' helminth secretory systems to an *in vitro* model to complement currently used phenotypic screens?

Will it be possible to reconstitute the host–parasite crosstalk using *ex vivo*/*in vitro* systems and apply a 'stress' situation on the parasite, mimicking the *in vivo* natural settings, to be integrated to anthelmintic screens?

Can the contribution of antisecretory actions of MLs and BZs to their clinical efficacy be quantified (or proven)?

Can we develop tools for functional genomics of nematodes that will enable characterization of PDM secretion biology and identify PDMs that perform essential roles in establishing and maintaining infection?

well as the mechanisms that control their release, will expand our list of targets that can be exploited for therapeutic intervention.

The host–parasite interface represents a conflict in which a state of 'disease tolerance' is negotiated to preserve mutual homeostasis. Disruption of this continuous molecular dialog may favor either the host or the parasite. Evidence from pharmacology and immunoparasitology research should therefore be reconsidered to inspire the search for novel ways to interfere in the communication between parasite and host. The prototype of almost every anthelmintic class was discovered in a screen using infected animals [31]. That strategy allowed the discovery of compounds that acted, at least in part, by preventing parasite-induced modulation of the host immune system. Switching to phenotypic screens of viability, development and/or motility as a strategy for anthelmintic discovery eliminated that possibility. We therefore recommend that efforts be made to incorporate screens for inhibition of PDM release in culture as a complement to existing phenotypic screens; technical challenges limit our ability to pursue this avenue, but expanding the scope of discovery programs to be able to detect such compounds seems worth the investment needed to bring them on-line.

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Declaration of Interests

The authors declare no competing interests.

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