Cysteine proteases as digestive enzymes in parasitic helminths

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Abstract

We briefly review cysteine proteases (orthologs of mammalian cathepsins B, L, F, and C) that are expressed in flatworm and nematode parasites. Emphasis is placed on enzyme activities that have been functionally characterized, are associated with the parasite gut, and putatively contribute to degrading host proteins to absorbable nutrients [1–4]. Often, gut proteases are expressed as multigene families, as is the case with Fasciola [5] and Hae-

monchus [6], presumably expanding the range of substrates that can be degraded, not least during parasite migration through host tissues [5]. The application of the free-living planarian and Caenorhabditis elegans as investigative models for parasite cysteine proteases is dis-

cussed. Finally, because of their central nutritive contribution, targeting the component gut proteases with small-molecule chemical inhibitors and understanding their utility as vaccine candidates are active areas of research [7].

Angiostrongylus

Cysteine proteases are essential in the degradation of host hemoglobin, the principal source of amino acids for many parasitic helminths. Angiostrongylus cantonensis and A. costaricensis are the etiological agents of abdominal angiostrongyliasis and eosinophilic meningoecephalitis, respectively [8, 9]. This nematode cycles between rodents and mollusks, and the parasite infects humans via the ingestion of raw vegetables and/or mollusks containing third-stage lar-

vae (L3). Only six cysteine protease sequences are available in the GenBank protein database for adult Angiostrongylus. The function(s) of these putative proteins is still unknown; however, they may play a role in immune system evasion and nutrient acquisition. In A. cantonensis, cathepsin B cysteine proteases may contribute to the invasion of the central nervous system during parasite–host interactions [10], penetration into the host’s gut wall [11], and feeding [12, 13]. Reverse-transcription PCR (RT-PCR) revealed that cysteine protease transcripts of A. cantonensis are present in both larval and adult worms [14]. In addition, a proteolytic enzyme
was localized in the intestine of juvenile and adult worms, indicating its probable contribution to feeding and nutrition [14, 15].

**Hookworm and *Haemonchus***

The two most prevalent hookworm species infecting humans are *Necator americanus* and *Ancylostoma duodenale*, with *A. ceylanicum* being found in certain areas [16, 17]. *Haemonchus contortus*, the barber's pole worm, which is common in pastures where cattle, sheep, and goats are farmed, is an appropriate comparator for human hookworms, being also hematophagous with a similar life history. Third-stage larvae (L3) are found in contaminated feces and are either ingested (*Haemonchus* and *A. duodenale*) or can actively invade the skin (hookworms). Adult parasites settle, mature, and mate in the abomasum (*Haemonchus*) or duodenum (hookworms). Adults are hematophagous, with blood being a rich source of nutrients, not least for the prodigious production of eggs: female parasites can produce 10,000–28,000 eggs per day ([18, 19] and references therein).

Considerable transcriptional effort is made by these nematodes into expressing a series of cathepsin B-like proteases that are found in the parasite's gut (esophagus and cecum) [6, 20–22]. These enzymes, among other protease classes [23], degrade blood proteins to absorbable nutrients [3, 4, 24–27] and are targets for small-molecule drug discovery (see below). Based on both classical biochemical and recent transcriptomic/genomic evidence, *N. americanus* and *A. ceylanicum* express six gut cysteine proteases [4, 22, 28], and *Haemonchus* as many as 22 individual proteases ([29] and references therein). In the latter case, further complexity is introduced by geographical variation in the molecular sequences and the activity of the individual proteases [30–33]. Differences in proteases are also apparent for the same parasite strains that are isolated from different hosts [33, 34]. Because they operate at the interface between host and parasite, the cysteine proteases of gastrointestinal nematodes have been tested as vaccine candidates with varying degrees of efficacy [24, 33, 35, 36]. In the case of *Haemonchus*, defining exactly which proteases represent vaccine candidates is complicated by the geographic and strain variations alluded to above.

**Trichuris and Ascaris**

*Trichuris* (whipworm) and *Ascaris* (giant roundworm), together with hookworm, comprise the three most prevalent soil-transmitted helminths, which collectively infect approximately 1.5 billion people [37]. *T. muris* (murine whipworm) and *T. suis* (porcine whipworm) are employed as models of *T. trichiura* (human whipworm). *A. lumbricoides* and *A. suum* are widespread roundworms of humans and pigs, respectively, with *A. suum* serving as a model [37, 38].

Drake and colleagues (1994) [39] first described a cysteine protease activity in soluble extracts of *T. muris* adult worms using a fluorogenic peptidyl substrate. They suggested a contribution by this enzyme in nutrition and/or invasion. A cysteine protease similar to cathepsin B was subsequently characterized in adult *T. suis* gut extracts and excretory/secretory products (ESP) using both fluorogenic peptide substrates and protein substrate gels [40]. Again, this enzyme was postulated to be involved in nutrition.

Hasnain and colleagues [41] showed experimentally that proteolytic activity in *T. muris* ESP is responsible for degrading host intestinal mucin. Whereas serine protease activity appeared to be the most active, the authors also suggested that cysteine proteases present in ESP contribute to disrupting the polymeric mucin network. High-throughput transcriptomics data indicate that a suite of cysteine proteases are expressed in *T. muris*, including legumain; calpains; and cathepsins B, L, F, and Z. [42]. One or more of these may be involved in
digestion. Recently, Santos and colleagues reported transcripts encoding gut-specific cysteine proteases in extracts of adult *T. trichiura* [43], and, at the proteome level, a number of these possessed immunomodulatory activity [44]. Few studies have focused on the functional characterization of *Ascaris* cysteine proteases. Early reports described a hemoglobinolytic cysteine protease activity in *A. suum* lysates [45, 46]. In addition, protease activity was described in *A. suum* intestinal lysates using a fluorescently (bodipy)-labeled casein substrate, although no significant inhibition with the cysteine protease inhibitors, E-64 and iodoacetamide, was measured [47]. In contrast, aspartic proteases seem to represent the largest group of *A. suum* proteases involved in digestion [47], whereas cysteine proteases are a relatively minor constituent. Finally, the predicted secretome of *A. suum* includes five cysteine protease sequences that may contribute to feeding and/or degradation of host tissue [48].

**Schistosoma**

Schistosomiasis, or bilharzia, is caused by several parasitic flatworms of the genus *Schistosoma*. It is endemic in over 70 tropical/subtropical countries and infects more than 200 million people. Infective larvae ( cercariae) are released from intermediate aquatic snail hosts, penetrate the outer and inner dermal skin layers, and then, as schistosomula, migrate to the blood capillaries. Schistosomula are carried to the lungs and subsequently to the liver before settling as mated male and female worms in the mesenteric (*S. mansoni* and *S. japonicum*) or bladder (*S. haematobium*) venules. Blood proteins, particularly hemoglobin, are an essential source of nutrients with which the female worms produce hundreds of eggs per day. A portion of the eggs laid exit the host via the intestine or bladder to continue the life cycle; however, many become trapped in the liver, gastrointestinal tract, or urinary tissues, where they can induce the immunopathology and fibrosis that are often associated with schistosomiasis [49].

Timms and Bueding (1959) [50] first described the presence of proteases with an acidic pH optimum in schistosome extracts. They suggested a role for these in degrading hemoglobin to free amino acids in the schistosome gut and also highlighted their potential as drug targets. Today, we know that an array of proteases are secreted into the schistosome gut, including papain-like cysteine proteases (clan CA, family C1) cathepsins B1, L1/F, L2, L3, and C (Table 1), as well as an aspartic protease, cathepsin D (Clan AA) [51–53].

The *S. mansoni* cathepsin B1 (SmCB1, also known as Sm31) was first described and characterized as a potential diagnostic protein based on potent immunogenicity in mice and humans [54–61]. The first described *S. mansoni* cathepsin L, SmCL1 [62–64], was later revealed to be more related to cathepsins F, which are distinguished from other cathepsins by various structural motifs and an N-terminal propeptide extension [1]. SmCL2, in contrast, is a defined cathepsin L, and because it was detected in the reproductive system of female schistosomes, it was speculated to function in increasing the fluidity of proteins in secretions within the ooviduct and activation of phenoloxidase, an enzyme involved in crosslinking eggshell proteins [65]. Bogitsh and colleagues [66] showed, using monospecific polyclonal antibodies, that both SmCL1/F and SmCL2 are located in the schistosome digestive tract and are likely digestive enzymes. More recently, Dvořák and colleagues [52] discovered a third and distinct *S. mansoni* cathepsin L, SmCL3, which is also localized in the gastrodermal cells.

Digestion of host blood proteins by schistosomes is performed in a bifurcated gut, which does not possess an anus, and hence the proteases and degraded contents are emptied by regular regurgitation into the blood stream of the host. The pH within the gut is in the range of 4.0 to 6.5 [67], which is important in regulating the digestive function of the gut proteases. Delcroix and colleagues [67] described how a “network” of proteases function best in acid pH and...
Table 1. Helminth parasite cysteine proteases and their functions.

| Parasite               | Protease | Proposed Function                                           | References                  |
|------------------------|----------|-------------------------------------------------------------|-----------------------------|
| **Nematodes**          |          |                                                             |                             |
| *Angiostrongylus*      | Cathepsin B | Tissue invasion                                             | [10]                        |
| *cantonensis*          | Cathepsin B1 |Degradation of hemoglobin and IgG                           | [14]                        |
| *Ancylostoma*          | Cathepsin B1 |Degradation of tissues                                       | [26, 151]                   |
| *caninum*              | Cathepsin B2 |Degradation of hemoglobin                                   | [26, 151, 152]              |
| *Haemonchus*           | Cathepsin B (multiple) |Degradation of hemoglobin, fibrinogen, collagen, and IgG    | [151]                       |
| *contortus*            | Cathepsin L |Degradation of hemoglobin, fibrinogen, collagen, and IgG; blood anticoagulation | [34]                        |
| *Necator*              | Cathepsins B2, B3, B4 and B5 |Degradation of globin fragments and gelatin | [4]                         |
| *Trichurus*            | Gut cysteine protease |Digestion                                                    | [42, 43]                    |
| *muris*                | Cathepsin B-like |Digestion                                                    | [40]                        |
| *Ascaris*              | Cathepsin B-like |Digestion                                                    | [47]                        |
| **Trematodes**         |          |                                                             |                             |
| *Schistosoma*          | Cathepsin B |Degradation of hemoglobin                                    | [153]                       |
| *haematobium*          |          |                                                             |                             |
| *Schistosoma*          | Cathepsin B |Tissue invasion                                              | [154]                       |
| *japonicum*            | Cathepsin C |Degradation of hemoglobin                                    | [155]                       |
| *Schistosoma*          | Cathepsin L1 |Degradation of hemoglobin; membrane turnover                | [64, 156]                   |
| *mansoni*              | Cathepsin B1 |Degradation of hemoglobin, albumin, and IgG                  | [56]                        |
| *Trichobilharzia*      | Cathepsin B |Degradation of myelin protein                                | [81]                        |
| *regenti*              |          |                                                             |                             |
| *Clonorchis*           | Cathepsins B1, B2, B3 and B4 |Degradation of gelatin, hemoglobin, albumin, fibronectin, and IgG | [82]                        |
| *sinensis*             | Cathepsins F4, F6 and F11 |Degradation of hemoglobin, albumin, collagen, and IgG | [86]                        |
| *Paragonimus*          | Cathepsin L-like |Degradation of extracellular matrix proteins and IgG     | [98, 158]                   |
| *westernmani*          | Cathepsin F |Possible role in egg production                              | [159]                       |
| *Hemoglobinase*        |          |Degradation of hemoglobin                                    | [100]                       |
| *Fasciola*             | Cathepsins B1 and B3 |Penetration of the intestinal wall                           | [104, 109]                 |
| *hepatica*             | Cathepsin B2 |Penetration of the intestinal wall; degradation of albumin and IgG | [104, 109, 160]            |
| *Cathepsin*            | Cathepsin C |Degradation of peptides                                      | [101]                       |
| *Cathepsin*            | Cathepsin L1 |Degradation of hemoglobin, IgG, and TLR3                    | [101, 102, 107, 108]        |
| *Cathepsin*            | Cathepsin L2 |Degradation of hemoglobin, collagen degradation, and IgG   | [101, 107]                 |
| *Cathepsin*            | Cathepsin L3 |Degradation of collagen                                       | [102]                      |
| *Cathepsin*            | Cathepsin L5 |Degradation of hemoglobin, fibronectin, laminin, and IgG    | [101, 105]                 |
| **Cestoda**            |          |                                                             |                             |
| *Taenia*               | Cathepsin L |Degradation of tissues and IgG                               | [110, 111]                 |
| *solium*               |          |                                                             |                             |
| *Taenia*               | Cathepsin L |Degradation of IgG and fibronectin                           | [112]                       |
| *pisiformis*           |          |                                                             |                             |
| *Echinococcus*         | Cathepsin L and B |Degradation of IgG, albumin, collagen, and fibronectin  | [118, 119]                 |
| *multilocularis*       |          |                                                             |                             |

Abbreviations: IgG, immunoglobulin G; TLR3, toll-like receptor 3.

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postulated that they either act simultaneously or in some specifically ordered manner to reduce protein macromolecules to absorbable peptides and amino acids. The pH of the gut is also important in facilitating denaturation of the protein substrates, unfolding their tertiary structure to make them more prone to hydrolysis [1, 67].

Immunolocalization studies suggest that the cysteine proteases are synthesized by the gastroduodenal cells and are secreted within vesicles into the gut lumen as inactive precursors or “zymogens.” Therefore, another important contribution of the gut’s low pH is the activation of these zymogens to their mature catalytic forms. Recombinant zymogen forms of the SmCL1/F, SmCL2, and SmCL3 effectively autocatalytically activate in vitro to mature enzymes only in low pH solutions [53]. SmCB1 is different in this respect, as the zymogen does not autocatalytically activate at low pH in vitro [53, 56].

An alternative mechanism for activating the SmCB1 zymogen was discovered, whereby a specific cleavage site at the junction between the propeptide and mature enzyme domain is processed by an asparaginyl endopeptidase (SmAE or legumain), a cysteine protease that is also found in the parasite gut [56, 68]. Sajid and colleagues [56] showed experimentally that SmAE could activate SmCB1 in vitro. However, paradoxically, when RNA interference (RNAi) methods were used to knock out SmAE activity in live schistosomula, SmCB1 was still fully processed and activated, suggesting that SmAE is not essential to activate SmCB1 in vivo [69]. Subsequently, X-ray crystal structures of the zymogen, mature enzyme, and an inactive intermediate form of SmCB1, resolved by Jilkova and colleagues [70], highlighted a further mechanism for protease activation. They showed that the propeptide of the SmCB1 contains a unique alpha-helix, alpha-3p. This helix is positioned upstream of the propeptide–mature enzyme juncture, protrudes from the enzyme core, and interacts with sulfated polysaccharides like dextran sulfate and heparin sulfate. Binding of sulfated polysaccharides to the alpha-3p was found to be essential for complete processing of the SmCB1.

The action of the cysteine and aspartic protease network in the schistosome gut would produce a plethora of peptides of various lengths that must be further broken down into free amino acids before they can be utilized in protein anabolism by the parasite. Dipeptidyl peptidase (DPP) I and III activities have been described in adult S. mansoni [71, 72]. Also, S. japonicum DPP I, a cathepsin C cysteine protease, was functionally expressed and shown to process dipeptides [71]. It is assumed that this enzyme plays a role in the hydrolysis of peptides alongside a leucine aminopeptidase (SmLAP) [73] to release free amino acids. Another possible role for DPP I is in assisting SmAE in the activation of proteases by trimming off propeptide amino acids that remain following processing by SmAE [1].

The redundancy of protease activation mechanisms (autocatalytic, trans activation/trimming, and sulfate polysaccharide triggered) within the schistosome gut probably reflects the central importance of the protease network in the nutrition, survival and reproduction of the parasite. Previous studies have demonstrated the validity of these enzymes as drug targets [74–76], with Abdulla and colleagues [74] demonstrating that treatment with the vinyl sulfone cysteine protease inhibitor, K11777, reduces worm and egg burden in mice. Furthermore, Ricciardi and colleagues [77] have shown that vaccination with recombinant SmCB1 induces strong protection against infection in a murine model of S. mansoni infection. The more we learn about the physicochemical, structural, and biological properties of these critical proteases, the better we will be able to design novel antischistosome drugs or vaccines.

**Trichobilharzia**

The schistosomes *Trichobilharzia regenti* and *T. szidati* can only complete their life cycles in specific birds. However, their cercariae can penetrate nonspecific host (mammalian) skin
(giving rise to cercarial dermatitis in humans) and migrate for a time through various tissues before dying [78]. An ortholog of SmCB1 has been characterized in the gut of both *T. regenti* and *T. szidati* schistosomula [79, 80] with at least 6 isoforms of TrCB1 (1.1–1.6) identified by PCR in the former species [81]. Interestingly, when recombinantly expressed, isoforms 1.1 and 1.4 efficiently degraded myelin basic protein but not hemoglobin, consistent with *T. regenti*’s tropism for and migration along the nervous system [81]. Like the purified zymogen of SmCB1 (above), the TrCB1.1 zymogen requires processing in *trans* (e.g., by SmAE) to become fully active, whereas the TcCB1.4 zymogen can autoprocess its own maturation. Like SmCB1 [60], TrCB1 is released from the parasite and may be useful as a serodiagnostic marker of infection [80].

**Clonorchis**

*Clonorchis sinensis*, the oriental liver fluke, is a food-borne parasite that inhabits the intrahepatic bile duct and causes clonorchiasis [82]. Humans are infected by eating raw or undercooked freshwater fish contaminated with *C. sinensis* metacercariae. Upon ingestion, the excysted metacercariae and the juvenile flukes invade the duodenum and migrate to the intrahepatic bile ducts, where they develop into adults [83].

Cysteine proteases (cathepsins B, F, and L and legumain) are expressed in different stages of the *C. sinensis* life cycle, with up-regulation occurring in adults [84–86]. Transcriptomic and proteomic analysis of *C. sinensis* tissues and ESP has shown that these cysteine proteases make up a major portion of the parasite’s total protease complement (especially cathepsin F proteases (CsCFs)) [86–89]. Furthermore, the cysteine proteases represent the most highly expressed protease family across multiple *C. sinensis* life stages associated with the mammalian host. This is indicative of *C. sinensis*’ dependency on these proteases during infection. Immunolocalization studies in adult parasites have shown that the cathepsins F and L proteases are mainly associated with the parasite’s intestinal wall and lumen [86, 90–92]. These findings and the fact that *C. sinensis* cysteine proteases can degrade an array of host proteins (e.g., hemoglobin, albumin, IgG, collagen, gelatin, fibronectin, and laminin) suggest functions in nutrition in addition to immune system evasion and tissue invasion [82, 90, 93]. The tandem expression and colocalization of cysteine protease inhibitors (cystatins) in this parasite suggests that the cysteine proteases are finely regulated [85, 94, 95]. The balance between cysteine proteases and cystatins [94, 95] could be susceptible to disruption by cysteine protease-inhibiting drugs.

**Paragonimus**

The lung fluke *Paragonimus westermani* causes paragonimiasis. Humans become infected with *P. westermani* after consuming raw or inadequately cooked crustaceans and/or wild boar meat that contain metacercariae. These metacercariae invade the duodenum and migrate through the abdominal wall before reaching the lungs and maturing as adult worms [96].

Cysteine proteases in *P. westermani* facilitate tissue invasion and immune evasion as well as nutrient acquisition [97, 98]. These enzymes are expressed in different developmental stages of *P. westermani*, especially in the secretome of adult flukes, consistent with their contribution to digestion of host proteins. Moreover, with at least 15 cysteine proteases present in the *P. westermani* secretome as well as their relatively high overall expression, cysteine proteases represent the major proteins secreted by this parasite [97, 99]. Despite a dearth of biochemical characterizations for *P. westermani* cysteine proteases, Choi and colleagues [100] have identified and characterized a hemoglobinase cysteine protease in adult worms. The application of a cysteine protease inhibitor, E-64, not only inhibits parasite cysteine proteases in vitro but also prevents *P. westermani* peritoneal invasion [98]. This suggests that
cysteine protease inhibitors may prove valuable as a novel chemotherapeutic approach for the treatment of paragonimiasis.

**Fasciola**

Recent genomic, transcriptomic, and proteomic analyses of the *Fasciola hepatica* life stages that infect the mammalian host have provided valuable information on the expression of key proteases potentially involved in host–parasite interactions [5, 101]. These data have also enabled clarification of the cysteine protease family structure, i.e., the cathepsins L represent a monophyletic family that contains five distinct clades, each with varying numbers of members: FhCL1 (6), FhCL2 (1), FhCL3 (5), FhCL4 (2), and FhCL5 (3). In contrast, the cathepsin B family consists of a monophyletic group with a single clade of 7 members [5].

These virulence-associated cysteine proteases show a strict temporal regulation in their expression during the parasite’s development in the mammalian host, which has furthered our understanding of their function [5]. For example, FhCL3 is highly expressed in newly excysted juveniles (NEJ) that initiate infection, but once the parasite has traveled across the intestinal wall, its expression is then down-regulated [5, 101]. Following this, the expression of FhCL1, FhCL2, and FhCL5 is up-regulated as the parasite migrates through the liver parenchyma and then takes up residence within the bile ducts [5, 101]. FhCL3 can accommodate small Gly residues in its active-site S3 pocket and bulky Pro residues in its S2 pocket, thereby having a potent ability to degrade collagen (which contains repeat Gly-Pro-X motifs). This feature allows the parasite to rapidly traverse the intestinal tissue and penetrate the Glisson’s capsule of the liver [102, 103]. NEJs at this stage are considered tissue feeders rather than blood feeders, so, besides the primary role of FhCL3 in tissue invasion, this enzyme may also be important for feeding [103]. Although FhCL2 has less collagenolytic activity relative to FhCL3 [102], it can still cleave collagen, and therefore, this enzyme likely assists migration throughout the host’s liver and into the bile ducts [104].

FhCL1, FhCL2, and FhCL5 can also digest host hemoglobin, suggesting they also have a key role in nutrient acquisition while the parasite is in the bile duct, where it is considered an obligate blood feeder [101, 105, 106]. The function of FhCL1 has been suggested to be primarily adapted to hemoglobin digestion [101]. The enzyme is the most predominant protease secreted by the adult fluke; it is liberated as a 37 kDa zymogen from the cecal epithelial cells lining the gut wall into the gut lumen, where it is activated to its 25 kDa mature form [101, 106]. In contrast to FhCL1, FhCL3 has a S2 subsite exhibits a marked preference for hydrophobic amino acids such as Phe, Leu, and Ala, which are the most common residues found in hemoglobin. The low pH of the parasite gut is important in facilitating the unraveling of the hemoglobin to allow FhCL1, which is active at both acid and neutral pH, to access and cleave peptide bonds [106].

The various FhCLs also cleave IgG and can prevent antibody-dependent cell cytotoxicity [107]. Furthermore, FhCL1 alters macrophage function via the cleavage of Toll-like Receptor 3 (TLR3) within endosomes; accordingly, the proteases have been proposed to aid the modulation or impairment of host immune responses as the parasite migrates through the tissues [107, 108].

Cathepsin B cysteine proteases are also important in the interaction of *F. hepatica* with its host [104]. Similar to FhCL3, FhB1, FhB2, and FhB3 are highly expressed in and secreted from NEJs but are then down-regulated once the parasite penetrates the liver [5], which would suggest specific functions in tissue penetration and feeding [109]. Other members of the FhCB family are not secreted and, consequently, may play housekeeping functions within the parasite’s tissues. Finally, a *F. hepatica* cathepsin C dipeptidase, which removes dipeptides from the
N-terminus of proteins, may further process peptides derived from the action of FhCL and FhCB proteases [101].

The importance of *F. hepatica* cysteine proteases in the invasion of tissues, the acquisition of nutrients, and in immunomodulation has made them key targets for the development of novel vaccines and drugs [104]. Indeed, vaccine studies in ruminants have demonstrated that recombinantly produced FhCLs offer substantial protection against experimental and field infections of liver flukes (reviewed in [104]).

**Cestoda**

In contrast to trematodes, cestode cysteine proteases have received relatively little attention, and some ambiguity remains regarding their precise functions. Nonetheless, attempts to functionally characterize the proteases identified have been made. For example, *Taenia solium* and *T. pisiformis* express cysteine proteases (particularly cathepsin L) that cleave human IgG [110–112]. Also, cysteine proteases in the ESP of *T. solium* were found to deplete CD4+ T cells and induce apoptosis in vitro [113, 114]. Although the degradation of immune-related proteins suggests a role in parasite defense against the host immune system, the degradation of these proteins could simultaneously provide a nutritional benefit to the parasite [115], particularly as IgG proteins have been identified in the cysts of various *Taenia* species [111, 116, 117]. Indeed, the uptake and proteolysis of IgG (and other serum proteins) by the cyst has been observed [116]. Perhaps the degradation of IgG not only protects the parasite from recognition by the host immune system but also provides a supply of amino acids to sustain itself within host tissues [115–117].

Recombinant cathepsin L from *T. pisiformis* degrades fibronectin and thus may aid the degradation of host tissues and consequently invasion [112]. Given the similarity of this cathepsin L to orthologous proteases in *C. sinensis*, *F. hepatica*, and *P. westermani* [110], further investigation is warranted to more fully understand its possible contribution to the degradation of host proteins. Similarly, a cathepsin L and two cathepsins B from *Echinococcus multilocularis* were found to degrade IgG, albumin, collagen, and fibronectin [118, 119]. Therefore, as with the *Taenia* species enzymes, the *E. multilocularis* cysteine proteases may be involved in nutrient acquisition, immunomodulation, and tissue invasion [118, 119] and, accordingly, may prove to be useful drug targets.

**Planaria and *C. elegans* as models for cysteine protease activity**

Because of the genetic intractability of most helminth parasites, it is difficult to characterize or interrogate protein function. Building the necessary tools, even those as basic as isolated marker mutations or genetic balancers, can take years, hindering the advancement of parasite research. One useful tool, transient RNAi, is variably effective in flatworm [120, 121] and roundworm parasites [122–124]. RNAi protocols differ widely but typically involve feeding, soaking, or electroporation with double-stranded RNA (dsRNA). Of these, electroporation is expensive and labor intensive and can cause parasite damage, e.g., to the larvae of *Brugia malayi* [125]. Furthermore, the ability to generate transgenic animals is dependent on parasite culture [126]. Long and complex life cycles involving more than one host may make the challenge of gene knockouts insurmountable for many parasites.

The use of free-living helminths to model parasitic nematodes and flatworms may compensate for many of these problems. *C. elegans* is a well-studied nematode with access to a great number of genetic tools. Transgenesis is routine, and transgenic animals can be easily enriched due to the ease of *C. elegans* culture. In addition, gene expression and protein localization can be observed with fluorescent reporters. Finally, gene editing using the CRISPR/Cas9 system is
now available for *C. elegans* [126]. *C. elegans* has also been used as an expression system for parasite proteins [127]. Expression of recombinant proteins can be used in vaccination studies, as these proteins are properly glycosylated and activated in the nematode. Because of the structural, developmental, and reproductive similarities between *C. elegans* and parasitic nematodes, the tools available to study *C. elegans* can be exploited to find new targets that may be important in parasites. Although *C. elegans* is likely a more effective model for clade V nematodes rather than either clades III or IV [128], there is still a high degree of similarity between these clades, so findings with *C. elegans* should not be discounted.

Although parasitism evolved independently multiple times in nematodes [129], all parasitic flatworms are derived from the monophyletic group “neodermata,” encompassing some 6,000 species [130]. Therefore, the possibility exists that a free-living flatworm can serve as a good model for many parasitic flatworms. The planarian flatworm, *Schmidtea mediterranea*, shares many important features with parasitic flatworms, especially *Schistosoma*. Both lay ectolecithal eggs [130], possess structural similarities in their protonephridia [131, 132], and contain a population of stem cells called neoblasts that are distributed throughout the body [133]. These neoblasts have been the source of most research on *S. mediterranea*, and their regenerative capabilities makes the propagation of *Schmidtea* fast, cheap, and easy. *S. mediterranea* also has a relatively small diploid genome that is fully sequenced and assembled [134]. Both large-scale whole-mount in situ hybridization (WISH) and RNAi can be performed with relative ease [135, 136], thereby making new and interesting targets much easier to find, in contrast to parasitic flatworms. Many of the genetic tools available for *C. elegans* are still underdeveloped for *S. mediterranea*, but planarian worms are among the best-characterized and studied flatworms and can serve as a potential model system for the less-tractable parasites.

Very little is known about the functions of proteases in *S. mediterranea*. Recently, cysteine proteases, along with aspartic proteases, have been identified as the major participants in protein digestion in the *S. mediterranea* gut [141]. These cysteine proteases include several cathepsins B and L. One specific cathepsin B protease, SmedCB, has been characterized, and its contribution to protein digestion seems to be redundant with cathepsin L proteases. The identification and importance of these two protease types in planarian digestion is similar to findings for parasitic flatworms, including schistosomes and *Fasciola*. As RNAi in *S. mediterranea* is highly efficient, this free-living worm could be used to characterize cysteine proteases that are difficult to knock down and study in parasitic flatworms. Finally, because parasite proteases are diverse and exhibit varying degrees of specialization that may not be directly relatable to or interrogable in free-living worm models, the use of worm models to functionally characterize parasite proteases may be restricted to those molecules that are more conserved in function.
Helminth cysteine proteases as drug targets: Proof-of-concept studies in small-animal models of schistosome and hookworm infection

Early work by Wasilewski and colleagues [76] demonstrated the potential utility of helminth cysteine proteases as drug targets. Specifically, they showed that daily intraperitoneal (i.p.) administration of approximately 100 mg/kg Mu-F-hF-FMK, a fluoromethyl ketone cysteine protease inhibitor, to mice harboring lung-stage or adult S. mansoni decreased worm burdens by 85% and 28%, respectively. The associated hepatic egg counts were decreased by 87% and 80%, respectively. This study was followed up 10 years later with the demonstration that the vinyl sulfone inhibitor, N-Me-piperazine-F-hF-vinyl sulfone phenyl (K11777), a preclinical candidate for Chagas disease (see the relevant chapter in this collection), could ameliorate a S. mansoni infection in mice. Specifically, when administered at 25 mg/kg twice daily i.p. for two weeks to target skin- and lung-migratory schistosome parasites, a >88% reduction in worm burden was achieved [74]. When administered for one week to mice harboring mature infections, worm burdens were reduced by 81% [74]. In both administration scenarios, the decrease in worm burdens was associated with pronounced egg burden reductions and associated liver and spleen pathologies. Importantly, in terms of the putative molecular target, the antiparasitic effect was correlated with a 90% decrease in the cathepsin B (SmCB1) activity of the parasite.

To pursue a target-based drug development program, crystal structures of SmCB1, bound to various small-molecule cysteine protease inhibitors, were generated [75]. Interestingly, the potency of inhibition of the recombinant SmCB1 [56] by these and other small-molecule inhibitors correlated with severity of the parasite’s (schistosomula) response phenotype in vitro [75]. Other strategies for SmCB1 inhibitor development have involved the use of small molecules derived from the propeptide (part of the SmCB1 zymogen that is removed during activation of the enzyme) [142] and a quantum mechanics scoring methodology to design and evaluate new inhibitors [143]. As attractive a target as SmCB1 is, it isn’t yet clear whether short courses of protease inhibitors would be significantly antiparasitic for eventual clinical use. This is in the context of the target product profile for new antischistosomal drugs that demands short-course (preferably single-dose) therapy [144] in order to facilitate mass drug administration (MDA), as is practiced for the current drug, praziquantel [145].

K11777 found a second life in a different animal model of helminth infection, namely the Golden Syrian hamster, which is a competent host for the hookworm A. ceylanicum. A single oral dose of 100 mg/kg provided near cure of infection [146]. Similar to the experiments with schistosomes, resident cathepsin B activity in the hookworm gut was decreased by over 95%. These efficacy and target-validation studies for K11777 and hookworm infection were supported by the use of a second and structurally unrelated cathepsin K inhibitor, namely, odanacatib (ODN) [147], which was withdrawn by Merck as a drug candidate for treatment of osteoporosis in September 2016. Specifically, a single oral dose of 100 mg/kg ODN decreased adult A. ceylanicum burdens by 73%, with a 51% reduction in the parasite’s cysteine protease activity. The efficacy measured was unexpected given ODN's exquisite specificity for cathepsin K over other cathepsins, including cathepsin B [148, 149], and it was suggested that ODN’s long plasma half-life was key to the pronounced efficacy measured. Accordingly, it was reasoned that combining K11777’s potency for cathepsin B with ODN’s excellent pharmacokinetic properties could lead to an inhibitor that is efficacious at a lower dose [147].

As with MDA for treatment of schistosomiasis, single oral dosing is the modus operandi of MDA programs that deliver essential antinematodal drugs [150]. In this context, the activities of K11777 and ODN in the hamster model of hookworm infection are encouraging, and the
search continues for cysteine protease inhibitors with improved on-target antiparasite efficacy at lower overall doses. Of importance to the success of any protease-inhibitor drug, however, will be an understanding of whether a substantial therapeutic benefit against the other, and often coendemic, soil-transmitted nematodes, namely, *Trichuris* and *Ascaris*, is also delivered.

### Key Learning Points

1. Cysteine proteases are key contributors to the digestion of host proteins by helminth parasites.
2. Helminth cysteine proteases are often expressed as multigene families.
3. Helminth cysteine proteases as drug and vaccine targets is an active area of research.

### Top Five Papers

1. Kasny M, Mikes L, Hampl V, Dvořák J, Caffrey CR, Dalton JP, et al. Chapter 4. Peptidases of trematodes. Adv Parasitol. 2009;69:205–97. Epub 2009/07/23. doi: S0065-308X(09)69004-7 [pii] 10.1016/S0065-308X(09)69004-7. PubMed PMID: 19622410.

2. Ranjit N, Zhan B, Stenzel DJ, Mulvenna J, Fujiwara R, Hotez PJ, et al. A family of cathepsin B cysteine proteases expressed in the gut of the human hookworm, *Necator americanus*. Mol Biochem Parasitol. 2008;160(2):90–9. Epub 2008/05/27. doi: S0166-6851(08)00094-7 [pii] 10.1016/j.molbiopara.2008.04.008. PubMed PMID: 18501979.

3. Cwiklinski K, Dalton JP, Dufresne PJ, La Course J, Williams DJ, Hodgkinson J, et al. The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. Genome Biol. 2015;16:71. Epub 2015/04/19. doi: 10.1186/s13059-015-0632-2 [pii]. PubMed PMID: 25887684.

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