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The role of cystatins in tick physiology and blood feeding

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Summary

Ticks, as obligate hematophagous ectoparasites, impact greatly on animal and human health because they transmit various pathogens worldwide. Over the last decade, several cystatins from different hard and soft ticks were identified and biochemically analyzed for their role in the physiology and blood feeding lifestyle of ticks. All these cystatins are potent inhibitors of papain-like cysteine proteases, but not of legumain. Tick cystatins were either detected in the salivary glands and/or the midgut, key tick organs responsible for blood digestion and the expression of pharmacologically potent salivary proteins for blood feeding. For example, the transcription of two cystatins named HISC-1 and Sialostatin L2 was highly upregulated in these tick tissues during feeding. Vaccinating hosts against Sialostatin L2 and Om-cystatin 2 as well as silencing of a cystatin gene from *Amblyomma americanum* significantly inhibited the feeding ability of ticks. Additionally, Om-cystatin 2 and Sialostatin L possessed strong host immunosuppressive properties by inhibiting dendritic cell maturation due to their interaction with cathepsin S. These two cystatins, together with Sialostatin L2 are the first tick cystatins with resolved three-dimensional structure. Sialostatin L, furthermore, showed preventive properties against autoimmune diseases. In the case of the cystatin Hlcyst-2, experimental evidence showed its role in tick innate immunity, since increased Hlcyst-2 transcript levels were detected in *Babesia gibsoni*-infected larval ticks and the protein inhibited *Babesia* growth. Other cystatins, such as Hlcyst-1 or Om-cystatin 2 are assumed to be involved in regulating blood digestion. Only for Bmcystatin was a role in tick embryogenesis suggested. Finally, all the biochemically analyzed tick cystatins are powerful protease inhibitors, and some may be novel antigens for developing anti-tick vaccines and drugs of medical importance due to their stringent target specificity.

Keywords

Cystatin; Cysteine proteases; Tick; Immunomodulators; Blood feeding; Midgut; Physiology

Introduction

Cystatins comprise a large superfamily of reversible and tight-binding inhibitors that act on/interact with papain-like cysteine proteases and legumains (Abrahamson et al., 2003).

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Cystatins are present in a wide range of organisms such as vertebrates, invertebrates, and plants as well as protozoa (Vray et al., 2002; Turk et al., 2008). They are involved in various vertebrate biological processes, e.g. in antigen presentation, immune system development, epidermal homeostasis, neutrophil chemotaxis during inflammation, or apoptosis (Reddy et al., 1995; Honey and Rudensky, 2003; Wille et al., 2004; Lombardi et al., 2005). As regulators of proteolysis, they are associated with cell/tissue homeostasis as well as proteolysis-related pathological conditions (Zavasnik-Bergant et al., 2008).

Cystatins are classified, according to certain sequence motifs and the number of conserved cystatin domains, into four subfamilies: the type 1 cystatins (also known as stefins), the type 2 cystatins, the type 3 cystatins (kininogens), and the type 4 cystatin-like proteins (fetuins, histidine-rich proteins) (Rawlings and Barrett, 1990). A recent classification was proposed that assigns each peptidase, or peptidase inhibitor family, with a unique identification number in the MEROPS classification system that is widely accepted in the proteolytic society (Rawlings et al., 2010). According to the MEROPS database, cystatins belong to the I25 family, and this family is further divided into the subfamilies I25A (stefins), I25B (type 2 cystatins and kinogens), and I25C (fetuins, histidine-rich proteins). In this review, we will refer to the different cystatins using the classical categorization and not the MEROPS code.

The first type 1 cystatin (stefin) was discovered in the cytosol of human polymorphonuclear granulocytes (Brzin et al., 1983) and named cystatin A (NCBI Accession no. P01040, Fig. 1). Shortly afterwards, the full protein sequence of cystatin B (NCBI: P04080) was determined, and this stefin was detected in the human liver (Ritonja et al., 1985) (Fig. 1). Both cystatins are potent and tight-binding inhibitors of papain, cathepsins L, S, and H, and they also inhibit cathepsin B, but with lower affinity (Turk et al., 2008). Stefins are cystatins of low molecular weight (about 11 kDa), and they possess a single cystatin domain. This domain consists of the conserved N-terminal glycine and two β -hairpin loops 1 and 2, with loop 1 possessing the conserved QXVXG motif (Fig. 1). These three cystatin regions form a wedge-like structure that interacts with the catalytic cleft of cysteine proteases (Grzonka et al., 2001). Stefins are mainly intracellular proteins without a signal peptide, lacking any carbohydrate side chains as well as disulfide bridges found in other cystatins (Abrahamson et al., 2003). Homologues of human cystatin A and cystatin B were discovered in different vertebrates such as mouse, rat, cat, pig, and cow and in invertebrates such as leeches where they regulate proteolysis (Lefebvre et al., 2004; Rawlings et al., 2006). Cystatins originating from parasites such as nematodes received immediate attention as candidate determinants of host-parasite interactions because of their potential interaction with cysteine proteases that play a role in immunity-mediated homeostatic mechanisms. Fg-stefin 1 from *Fasciola gigantica* (NCBI: ACS35603) inhibited cathepsin B, L, and S activities and was suggested to protect the intestine and tegumental surface of the parasite against extracellular proteolysis (Tarasuk et al., 2009). Sm-cystatin from *Schistosoma mansoni* (NCBI: AAQ16180) successfully inhibited papain (Morales et al., 2004) and might play a role in the digestion of hemoglobin since it is localized in the parasite's gut (Wasilewski et al., 1996).

The prototypical type 2 cystatin was isolated in 1968 from chicken egg white (Fossum and Whitaker, 1968). This cysteine protease inhibitor interacted with papain, ficin, and cathepsins B and C (Sen and Whitaker, 1973; Keilová and Tomásek, 1975). The name 'cystatin' was given to this protein to indicate its function as a cysteine protease inhibitor (Barrett, 1981). The differences in the protein sequence of chicken cystatin and human stefin A accounted for significant differences in their resolved structures. Like stefins, type 2 cystatins possess a single cystatin domain that bears the conserved N-terminal glycine and the QXVXG segment of loop 1 (both sequence motifs are shown in the alignment of Fig. 1, and the cystatin domain is seen in Fig. 2A). Unlike stefins, type 2 cystatins also possess a conserved PW dipeptide in the β -hairpin loop 2 as shown in the case of human cystatin C

(NCBI: P01034). They are secreted proteins of 13–15 kDa molecular weight with a signal peptide and two intracellular disulfide bonds (Turk et al., 2008; Zavasnik-Bergant et al., 2008) (Fig. 1). Type 2 cystatins are generally non-glycosylated proteins (Dickinson, 2002); however, there are exceptions, for example, human cystatins F and E/M are glycosylated (Sotiropoulou et al., 1997; Halfon, et al., 1998). Besides inhibiting C1 proteases, human cystatin C is also able to inhibit legumain/asparaginyl endopeptidases with a second conserved SND inhibitory domain (Fig. 1) located between the conserved N-terminal glycine and loop 1 (Zavasnik-Bergant et al., 2008).

In parasites, type 2 cystatins are widely studied rather than stefins. For example, in nematodes, onchocystatin of *Onchocerca volvulus* was discovered in the cuticle of some larval developmental stages, the adult stages and the eggshell of microfilariae (Lustigman et al., 1991, 1992). Onchocystatin inhibited human cathepsins L and S, suggesting that this cystatin modulates the moulting of the larval stages while it is also involved in developing microfilariae in the uterus. Furthermore, this cystatin also showed strong immunomodulatory properties; it affected the human peripheral blood mononuclear T cell proliferation, the induction of TNF- α , nitric oxide, and IL-10, and it was responsible for the downregulating activities of the MHC II complex and CD86 molecules (Schönemeyer et al., 2001; Hartmann and Lucius, 2003; Schierack et al., 2003).

Type 3 cystatins are also known as kininogens; they are large (60–120 kDa), secreted multidomain proteins of three repeated type 2-like cystatin domains, where only the last two domains account for the inhibitory activity of the protein (Ohkubo et al., 1984; Müller-Esterl et al., 1985). These cystatins are found in extracellular fluids, they are usually glycosylated, and they possess eight disulfide bridges. Kinogens are involved, for example, in the protection against leaking lysosomal cysteine proteases or proteases derived from invading microorganisms; they also coordinate adaptive immunity (Scharfstein, et al., 2007). Fetuins and histidine-rich glycoproteins from the type 4 cystatin subfamily are also secreted proteins, but they lack the cystatin-inhibitory properties; thus they are only cystatin-like proteins possessing two tandem cystatin domains (Brown and Dziegielewska, 1997).

Ticks, as obligate hematophagous ectoparasites, greatly impact animal and human health because they transmit various pathogens worldwide. Family 1 and family 2 cystatins have been reported in various species of soft and hard ticks. Tick control measures are difficult (e.g. acaricide applications), but new strategies such as developing anti-tick vaccines are being implemented (de la Fuente et al., 2007). These vaccines are based on tick antigens that have immunomodulatory properties and interact with the vertebrate immune system. In this review, we present all currently described cystatins from hard and soft ticks, and we review their role in tick physiology. We describe their inhibitory activities on various cysteine proteases and their host immunomodulatory function during tick feeding. Particular emphasis is given to any demonstrated potential of tick cystatins as candidate tick antigens towards anti-tick vaccine development, and to their potential for developing novel pharmacological applications.

Type 1 cystatins in ticks

Tick cystatins are divergent from all the other described vertebrate, invertebrate, and plant cystatins. For example, in comparison to the prototypical human cystatins A, B and C, tick cystatins of type 1 and 2 only show up to 37% and 25% amino acid identity, respectively (Fig. 1). Currently, all known type 1 tick cystatins were discovered from five different hard tick species from four genera. All these stefins were described from transcriptome sequencing projects, and they are mostly found in tick salivary glands or midguts. Only two

stefins have been biochemically analyzed to an extent that we can discuss their role in tick physiology.

Genus *Rhipicephalus* – Bmcystatin of *R. microplus* and a stefin of *R. sanguineus*

The first biochemically characterized type 1 cystatin in ticks was Bmcystatin (NCBI: ABG36931) from *Rhipicephalus (Boophilus) microplus* (Lima et al., 2006). This intracellular stefin was found in tick fat body by cDNA library screening, and it was also detected in tick ovaries by PCR (Table 1). Anti-Bmcystatin antibodies also recognized this protein in a salivary gland extract by Western blot. Bmcystatin was highly similar to stefin B-like proteins in its amino acid sequence, its molecular weight of 11 kDa, and its isoelectric point (pI) of 5.7 (Lima et al., 2006; Zavasnik-Bergant et al., 2008). It also bears a rather modified cystatin domain, typical for stefins. More specifically, Bmcystatin possesses the N-terminal glycine and the QXVXG segment of loop 1 (typical motifs in all cystatins), but not the C-terminal PW motif (Fig. 1). Furthermore, Bmcystatin has a 70% sequence identity with a cytoplasmic salivary protein of *I. scapularis* (NCBI: AAY66864) that was the first type 1 cystatin identified in the sialome of *I. scapularis* (Lima et al., 2006; Ribeiro et al., 2006) (Fig. 1). Lima et al. (2006) suggested a role of Bmcystatin in the embryogenesis of *R. microplus* because this cystatin was found to inhibit, besides human cathepsin L, a vitellin-degrading cysteine endopeptidase of *R. microplus* (VTDCE).

Within the *Rhipicephalus* genus, a second type 1 cystatin was recently identified (NCBI Accession no. ACX53850), a stefin of *R. sanguineus* (Anatriello et al., 2010). This cystatin was found in the sialotranscriptome of *R. sanguineus* (Table 1) and only a 3'-truncated cDNA sequence of this protein was disclosed in public databases (Fig. 1). Nevertheless, this cystatin shows 87% amino acid sequence identity with the type 1 cystatin of *R. microplus*, and it also possesses the conserved cystatin domain of loop 1 (QXVXG). The N-terminal glycine of the conserved cystatin domain, however, appears more C-terminal in the amino acid sequence compared with all other described type 1 tick cystatins to date; the same exception is true for the *I. scapularis* stefin (NCBI: AAY66864) with a glycine residue at position 15 in the protein sequence (Fig. 1). The function of the *R. sanguineus* stefin still remains unknown.

Genus *Haemaphysalis* – Hlcyst-1 of *H. longicornis*

Apart from Bmcystatin of *R. microplus*, another midgut stefin, named Hlcyst-1, of *Haemaphysalis longicornis* (NCBI: ABZ89553) was also biochemically analyzed for its inhibitory activity (Zhou et al., 2006, 2009). This protein was highly similar to Bmcystatin with its predicted molecular weight of 11 kDa and a pI of 5.5 (Table 1). The protein showed a 79% amino acid identity with Bmcystatin including an identical cystatin domain of loop 1 (Fig. 1). Hlcyst-1 was localized in epithelial cells of the tick midgut alongside with HICPL-A, a cathepsin L-like cysteine protease of *H. longicornis* (Yamaji et al., 2010). HICPL-A seems to play a role in the digestion of host hemoglobin in ticks since it is able to degrade bovine hemoglobin (Yamaji et al., 2009a). Hlcyst-1 efficiently inhibited the hemoglobinolytic activity of HICPL-A, and both gene transcripts were upregulated during blood feeding of *H. longicornis* with their strongest expression at 48 h in the midgut cells (Yamaji et al., 2010). Thus, Hlcyst-1 seems to function as a regulator for digesting blood in ticks. Recombinant Hlcyst-1 was also able to effectively inhibit the cysteine proteases papain and mammalian cathepsin L (Zhou et al., 2009; Yamaji et al., 2010). Additionally, Hlcyst-1 and Bmcystatin showed a minor inhibitory activity towards cathepsin B, similar to human stefin A and B (Lima et al., 2006; Zhou et al., 2009). This may be explained by the steric hindrance caused by an occluding loop in the cathepsin B tertiary structure resulting in a significantly restricted access to the enzyme active site for the protease inhibitors (Stubbs

et al., 1990). Hlcyst-1 also inhibited cathepsin H in low levels that were comparable to the minor inhibitory activity towards cathepsin B (Yamaji et al., 2010).

Genus *Dermacentor* – DvM602 of *D. variabilis*

Another divergent type 1 cystatin DvM602 (NCBI: ACF35512) was identified in the midgut transcriptome of the hard tick, *Dermacentor variabilis* (Anderson et al., 2008). Apart from the *I. scapularis* stefin (NCBI: AAY66864), DvM602 shows a high amino acid sequence similarity of 78–86% to the other described cystatins (Fig. 1). Similar to all other type 1 cystatins, DvM602 possesses neither a signal peptide nor the PW motif at the C terminus, but it does possess the conserved cystatin domain of loop 1 (QXVXG loop). The inhibitory function of DvM602, however, still remains undetermined.

Special characteristics of tick type 1 cystatins

From all the type 1 cystatins described in this review, the cytoplasmic cystatin of *I. scapularis* differs remarkably in its amino acid composition in that it only scores a maximum of 67% identity (Fig. 1). The glycine residue of the putative cystatin domain is not located at the beginning of the N terminus which is the case for the other tick cystatins; it is rather shifted towards the C terminus of the protein. More interestingly, unlike all other type 1 tick cystatins, the *I. scapularis* stefin does not contain any cysteine residue (Fig. 1) as it is the case for the prototypical type 1 human cystatin A (NCBI: P01040) (Machleidt et al., 1983). All other tick stefins possess at least two cysteine residues in their amino acid sequence. Cysteine residues typically occur in type 2 cystatins to form disulfide bridges that support the stability of the protein's tertiary structure; however, type 1 cystatins are defined to lack these bonds (Turk et al., 2008). Type 1 cystatins are known to be primarily intracellular cytoplasmic proteins, and the intramolecular formation of disulfide bridges of cytosolic proteins is rather rare compared to secreted proteins due to the reducing environment of the cytoplasm and the lack of enzymes in this cellular compartment that promote cysteine pairings (Kadokura et al., 2003). Oxidative stress may lead to the formation of disulfide bonds within cytoplasmic proteins; however, these bonds are often nonspecific and cause irreversible damage to the proteins (Berlett and Stadtman, 1997). Human cystatin B (NCBI: P04080) was also found to possess one cysteine residue, and this stefin was able to dimerize by an intermolecular disulfide bridge between the cysteine residues of two monomers (Lenar et al., 1986) (Fig. 1). Dimerization, however, led to a loss of its inhibitory activity.

Type 2 tick cystatins

Most likely due to their secretory nature, type 2 cystatins have been intensively studied in ticks compared to stefins. In recent years, several studies in vector control research have focused on the pharmacological and immunomodulatory properties of saliva in order to understand arthropod blood feeding as well as pathogen transmission. Within this context, it is not surprising that most of the cystatins studied in ticks are secreted type 2 cystatins. A total of 14 type 2 cystatins and two cystatin-like proteins (from *Ornithodoros coriaceus*) from hard and soft ticks are currently described in the literature, and of these 9 cysteine protease inhibitors are also functionally characterized at the biochemical level (Table 1).

Genus *Ixodes* – sialostatins of *I. scapularis*

Among the secreted cysteine protease inhibitors from ticks, sialostatin L (SL, NCBI: AAM93646) from the salivary glands of *I. scapularis* was the first type 2 cystatin found in ticks by a sialotranscriptome study (Valenzuela et al., 2002). The name SL derives from its ability to inhibit cathepsin L with a K_i in the low pM range (Kotsyfakis et al., 2006). A few months later, sialostatin L2 (SL2, NCBI: AAY66685), a cystatin that also inhibits cathepsin L with similar affinity as SL, was described in the sialome of *I. scapularis* and characterized

at the biochemical level (Ribeiro et al., 2006; Kotsyfakis et al., 2007). Both cystatins showed high similarities in sequence (75% identity, Fig. 2), molecular weight (~12 kDa), and pI (pI~5) (Valenzuela et al., 2002; Ribeiro et al., 2006) (Table 1). The proteins, however, are encoded by two different genes as shown by NCBI BLAST analysis of their cDNA transcripts against *I. scapularis* genome (Kotsyfakis et al., 2007).

Similar to all type 2 cystatins, such as human cystatin C, both sialostatins are secreted proteins that possess a signal peptide as well as the conserved N-terminal glycine, the hairpin loops 1 and 2, and two disulfide bridges (Kotsyfakis et al., 2006, 2007) (Figs. 1 and 2A). Unlike cystatin C, the conserved PW segment of loop 2 is absent in both sialostatins, as for all described tick stefins. The QG residues, however, of the SL2 loop 2 match identically with the vertebrate cystatin C sequence and accordingly the PW motif is replaced by an NL dipeptide in SL2 (Figs. 1 and 2A). This difference in the amino acid sequence of loop 2 for the sialostatins suggested a divergent inhibitory activity for both sialostatins and indeed, both sialostatins do not interact with cathepsin B and H even not in 3 orders of magnitude excess. This inability of sialostatins to inhibit cathepsins B and H may be attributed to their unique amino acid sequence (Kotsyfakis et al., 2006, 2007). Björk et al. (1996) working with mutants of human cystatin C demonstrated a reduced cystatin affinity for both cathepsins B and H if a single amino acid substitution in the PW segment occurred. Finally, the conserved SND motif that is present in all the cystatins that inhibit legumain, such as cystatin C (Fig. 1, indicated with an asterisk), was absent in both sialostatins and as a result, they did not inhibit legumain (Kotsyfakis et al., 2006, 2007). In crystallography experiments, SL formed a domain-swapped dimer, typical of some vertebrate cystatins, while SL2 crystallized as a monomer (Kotsyfakis et al., 2010) (Fig. 2A). However, SL seems to appear in its monomeric structure in solution rather than as a dimer, suggesting that these dimers were formed in the process of crystal formation under specific experimental conditions. The major structural differences between both sialostatins appear in their N terminus. The N-terminal conformation of SL is similar to known structures of other type 2 cystatins (extended free N-terminal trunk), whereas the N terminus of SL2 is packed against the β -sheet (Kotsyfakis et al., 2010). These differences in the N-terminal segments of both sialostatins mediate their different binding affinities to their protease targets as indicated by mutagenesis experiments where the N terminus of SL2 is truncated. Both sialostatins inhibited cathepsin L with a high affinity. The tight binding interaction of SL2 and cathepsin L is shown in a docking experiment in Fig. 2B. Furthermore, high affinity of both sialostatins was experimentally confirmed for cathepsin V and papain (Kotsyfakis et al., 2006, 2007, 2010). On the other hand, differences in the inhibition affinity for different cysteine proteases were also revealed between the two cystatins with SL being a potent inhibitor of cathepsins C and S, whereas SL2 showed a much weaker interaction with these cysteine proteases.

Analyses to uncover the function of the different sialostatins showed that SL activity against cathepsin S affected the maturation of dendritic cells by inhibiting the invariant chain processing in dendritic cells. In addition, SL inhibited the antigen-specific CD4⁺ cells proliferation and the proliferation of cytotoxic T cells (Kotsyfakis et al., 2006; Sá-Nunes et al., 2009). SL was also able to inhibit neutrophil migration during acute inflammation and showed a preventive potential against autoimmune diseases (Kotsyfakis et al., 2006; Sá-Nunes et al., 2009).

Sialostatins are important for the blood feeding of ticks; in particular SL2 is highly upregulated in the salivary glands during *I. scapularis* feeding (Kotsyfakis et al., 2007). Ticks with SL2 and SL genes silenced were not able to feed on rabbits (40% of all ticks), or they were significantly less efficient in taking up blood (Kotsyfakis et al., 2007). Interestingly, a much lower feeding success was also observed in normal ticks that tried to

feed on animals previously exposed to SL2-silenced ticks (or in other words upon second exposure of the same animal that was previously exposed to SL2-silenced ticks). This suggested powerful immunomodulatory properties of sialostatins that were further verified by increased inflammation in the animals exposed to SL2-silenced ticks. Accordingly, SL2 was also utilized to vaccinate animals, and this vaccination led to an early rejection of ticks from the animals' feeding site or a reduced ability of ticks to feed on these animals for a prolonged time (Kotsyfakis et al., 2008). These phenomena might be attributed to the inhibition of the cathepsin L activity by SL2, although this needs to be confirmed experimentally.

Since ticks are important vectors of human and animal diseases worldwide, both sialostatins were also tested in a mouse model for their role in transmission of *Borrelia burgdorferi* s.l., the Lyme disease agent. Co-injection of SL with *Borrelia* had no effect on the number of spirochetes detected (4 days post co-injection) into the skin of mice. In contrast, SL2 co-injections into the mice skin resulted in an about six-fold increase in spirochete numbers (Kotsyfakis et al., 2010). Moreover, SL2 was not able to stimulate *Borrelia* proliferation in vitro, and no direct interaction of this cystatin with the parasites was observed. Thus, *B. burgdorferi* s.l. seems to take advantage of the immunosuppressive and vertebrate host modulatory mechanism induced by the presence of SL2 in the inoculation site upon its establishment in the skin of a mouse.

Beyond these two secreted sialostatins and the cytoplasmic stefin from *I. scapularis* that are published in the literature, 26 more *I. scapularis* cystatins are currently available in the NCBI database. These cysteine protease inhibitors originate from the recently completed *I. scapularis* genome (see at VectorBase for the full genome: <http://iscapularis.vectorbase.org/>).

Genus *Ixodes* – a cystatin of *I. ricinus*

One of the first discovered tick cysteine protease inhibitors at the sequence level was a putative secreted midgut cystatin of *Ixodes ricinus* (NCBI: CAD68002) (Jacot, 2003). Although this 14-kDa cystatin represented a typical member of the 125B family bearing a classical cystatin domain, it does not show a high similarity (a maximum of 33% amino acid identity) with the sialostatins of *I. scapularis* (Fig. 1), a closely related tick species to *I. ricinus*. In contrast to sialostatins, this cystatin possesses the classical PW domain of loop 2. Anti-cystatin antibodies were used to localize the cystatin in the midgut compartments, especially to detect the cells that secrete the protein during tick blood feeding (Jacot, 2003). However, immunohistological analysis did not clearly indicate the cystatin localization because almost all midgut compartments (lumen, cytoplasm, organelles, nucleus) were recognized by the antibodies. Furthermore, these antibodies were used to analyze the cystatin's interaction with *Borrelia* with no parasite recognition detected. Thus, the cell compartments of the midgut, the tissue specificity, as well as the function and role of the cystatin in tick feeding (or pathogen transmission) remain unclear.

Genus *Amblyomma* – a cystatin of *A. americanum* and *A. variegatum*

Based on the amino acid sequence of SL, another cystatin was discovered in the salivary glands and midgut of *Amblyomma americanum* (Karim et al., 2005). Only a partial sequence of this type 2 cystatin was determined, due to the design of the cloning primers based on the SL gene that did not cover the full length of the *A. americanum* cystatin gene. This partial sequence is 99% identical to the *I. scapularis* SL amino acid sequence. This cystatin possesses the conserved cystatin domain and the disulfide bridges, but it is missing the N-terminal glycine due to the above-mentioned gene cloning artifact (Fig. 1). Karim et al. (2005) tested the immunomodulatory role of this tick cystatin by silencing the cystatin

gene in ticks. Gene transcript abundance in the salivary glands and the midgut of silenced ticks decreased about 90% after 24 h of tick attachment to rabbits and increased again after 9 days of tick feeding. Only 20% of the silenced ticks completed their meal within the usual blood feeding duration. Half of the ticks gained less weight (with an average less than 50% weight compared to the weight gained by the control group), and they also fed more than 9 days on the host, compared to nonsilenced ticks of the control group. Ten percent of the ticks detached already after 24 h, and the remaining 20% of ticks died during feeding on the rabbits. As shown for SL, a strong host immune response was observed when female ticks were allowed to feed on rabbits that were previously exposed to silenced ticks (second exposure of rabbits to ticks). One third of all ticks detached after 24 h, and half of the remaining ticks died while feeding. These experiments demonstrated the powerful immunosuppressive characteristics of salivary cystatins that are conserved in two different tick species.

Another type 2 cystatin of the *Amblyomma* genus (NCBI: DAA34288) was recently discovered in the sialome of the tropical bont tick, *A. variegatum* (Ribeiro et al., 2011). This cystatin clearly represented a member of the type 2 cystatin family, although only 3'-truncated transcripts of this cystatin gene were sequenced (Fig. 1). As a result, the amino acid residues of loop 2 and the last cysteine at the C terminus are still unknown and thus, it remains unclear whether or not this new cysteine protease inhibitor possesses the PW motif of loop 2. Moreover, this cystatin shares only a 34% amino acid sequence identity with the cystatin from *A. americanum* (Fig. 2) and thus, we cannot presume that it holds similar immunomodulatory properties to the secreted cystatins of *A. americanum* and *I. scapularis*.

Genus *Rhipicephalus* – two cystatins of *R. sanguineus*

A type 2 cystatin of *R. sanguineus* (NCBI: ACX53922) shows the highest protein sequence similarity with that of *A. variegatum* (Fig. 1). This *R. sanguineus* cystatin and a second secreted cystatin from the same tick species (NCBI: ACX53862) were discovered by sequencing a salivary gland cDNA library of *R. sanguineus* (Anatriello et al., 2010); in this library the already described stefin of *R. sanguineus* was also found (NCBI: ACX53850, see above). Both *R. sanguineus* cystatins are not similar in their protein sequence, but in comparison to the sialostatins and the *Amblyomma* cystatins, the two cysteine protease inhibitors possess the classical PW segment of loop 2 (Fig. 1). Furthermore, the first secreted cystatin of *R. sanguineus* (NCBI: ACX53922) seems to have unique primary structural characteristics relative to all the described cystatins in this review due to its long N-terminal domain (of about 24 amino acids) in comparison with other cystatins. The same is true for its five cysteine residues, unlike the classical four cysteine residues of all other cystatins shown in Fig. 1. Further analysis is necessary to identify any unique and maybe novel functions of this cysteine protease inhibitor as a consequence of its divergent protein sequence.

Genus *Dermacentor* – DvM334 of *D. variabilis*

Apart from the type 1 cystatin (NCBI: ACF35512, see above section about type 1 cystatins), two more secreted cystatins, namely DvM334 (NCBI: ACF35514) and DvM226 (NCBI: ACF35513) were identified in the midgut transcriptome of *D. variabilis* (Anderson et al., 2008). Only 5'-truncated cystatin cDNAs were sequenced for these cystatins and because of their 100% sequence identity (apart from one substituted amino acid), only DvM334 is presented in this review (Fig. 1). The highest similarity of DvM334 protein sequence (76%) was shown with that of the cystatin of *R. sanguineus* (NCBI: ACX53862). Although its secretion cannot be confirmed due to the missing N terminus, DvM334 bears classical type 2 cystatin sequence motifs including the cysteine protease binding sites loop 1 (QXVXG

motif) and 2 (PW motif) and the expected disulfide bridges due to the four cysteines in the amino acid sequence of this cystatin.

Genus *Haemaphysalis* – Hlcyst-2, Hlcyst-3, and HISC-1 of *H. longicornis*

Three more type 2 cystatins, namely Hlcyst-2 (NCBI: ABC94582), Hlcyst-3 (NCBI: ABZ89554), and HISC-1 (NCBI: BAI59105) were found in different tissues of *H. longicornis* (Zhou et al., 2006, 2010; Yamaji et al., 2009b). All three cystatins were expressed in different tick tissues; Hlcyst-2 and Hlcyst-3 were detected predominantly in the midgut and HISC-1 in the salivary glands of *H. longicornis*. All *H. longicornis* cystatins, especially HISC-1, differed in their protein sequence from other secreted hard tick cystatins (Fig. 1). HISC-1 showed many differences in its sequence concentrated close to loop 1 (positions 28–39) and between loop 1 and 2 (positions 61–95) when compared to the other secreted cystatins; it also possessed only three cysteine residues. Yamaji et al. (2009b) presented a possible signal peptide of 28 amino acids for HISC-1, however, although trying to verify the stated signal peptide using the same SignalP prediction server as Yamaji et al. (2009b) did, we cannot confirm their statement. Nevertheless, all 3 cystatins showed the typical sequence characteristics for type 2 cystatins.

Biochemical analyses confirmed that all cystatins are potential inhibitors of papain and cathepsin L, while Hlcyst-2 also inhibited cathepsin H (Zhou et al., 2006, 2010; Yamaji et al., 2009b, 2010). Although all cystatins had a similar inhibition profile for C1 cysteine proteases, they differed in their role in tick physiology. Hlcyst-2 was able to counteract the degradation of hemoglobin by inhibiting HICPL-A as already described for Hlcyst-1 (NCBI: ABZ89553), the intracellular cystatin of *H. longicornis* (Yamaji et al., 2010). This inhibitor was present in all tick developmental stages including the eggs, and its relative expression gradually increased during the tick's life cycle from larvae to adults. The Hlcyst-2 gene transcription in the midgut increased until 4 days of tick feeding followed by a decrease in the transcription level until the end of the blood meal (Zhou et al., 2006). Apart from its suggested role in the blood digestion, Hlcyst-2 might be involved in tick innate immunity. Evidence for this hypothesis was given by experiments demonstrating the up-regulation of cystatin transcripts after the injection of LPS into adult *H. longicornis* ticks. This upregulation was also observed after the infection of larval ticks with *Babesia gibsoni* (Zhou et al., 2006). Additionally, a significant inhibition of *B. bovis* growth and changes in the parasite morphology occurred when the parasites were exposed to Hlcyst-2 in an in vitro parasite culture.

Similar characteristics and functions are suggested for Hlcyst-3, which might regulate longipain, a papain-family cysteine protease identified in the midgut of the tick *H. longicornis* (Tsuji et al., 2008). Longipain plays a role in pathogen transmission (Tsuji et al., 2008), and thus this cystatin might be involved in the tick's anti-pathogen defensive mechanism (Zhou et al., 2010). HISC-1, found mainly in the salivary glands of *H. longicornis*, was localized in the type II acini cells (Yamaji et al., 2009b) – cells that secrete pharmacologically active compounds into the host. The HISC-1 transcript levels were highly upregulated within the first 24 h of tick blood feeding, and afterwards, the gene expression decreased gradually. In contrast, the transcript levels of the same gene were consistently downregulated in *H. longicornis* midguts.

Genus *Ornithodoros* – Om-cystatin 1, Om-cystatin 2 of *O. moubata*, and cystatins from *O. coriaceus* and *O. parkeri*

Limited information is available about cystatins from soft ticks, and the only cysteine protease inhibitors studied are type 2 cystatins from the genus *Ornithodoros*. Only Om-

cystatin 1 (NCBI: AAS01021) and Om-cystatin 2 (NCBI: AAS55948) of *O. moubata* were biochemically analyzed for their inhibitory activity towards papain like cysteine proteases and their role in the tick physiology (Grunclová et al., 2006; Salát et al., 2010). Om-cystatins 1 and 2 were clearly classified as type 2 cystatins because they contain a signal peptide, the conserved cystatin domain, and two possible disulfide bridges similar to most secreted cystatins from hard ticks (Fig. 1); they are about 50% similar in their protein sequence to the other type 2 cystatins. Om-cystatin 2 is the second secreted cystatin after SL2 whose structure was resolved and shown to adopt the typical cystatin fold (Salát et al., 2010). The closest structural homolog of Om-cystatin 2, at the primary level, was SL2, and thus the crystal structure of Om-cystatin 2 was determined by molecular replacement methodology using the SL2 structure [PDB code 3LH4, root mean square deviation (RMSD): 1.25 Å, Fig. 2A]. Lower structural similarities of Om-cystatin 2 were found with vertebrate type 2 cystatins such as human cystatin F (PDB 2CH9; RMSD ~2.0 Å), chicken egg-white cystatin (PDB 1YVB; RMSD ~2.6 Å), and human cystatin D (PDB 1RN7; RMSD ~2.8 Å).

Both Om-cystatins 1 and 2 were identified in the midgut by PCR and indirect immunofluorescence microscopy, and Om-cystatin 2 was also detected in the salivary glands, Malpighian tubules, and ovaries by PCR. In all tissues, both cystatin transcript levels were strongly downregulated after one day of blood feeding, however the abundance of Om-cystatin 1 in the gut was much lower compared to Om-cystatin 2 (Grunclová et al., 2006). Om-cystatin 2 was detected in type 2 secretory cells of the salivary glands (Grunclová et al., 2006), and it was also released into the midgut lumen of ticks with partially digested blood. In the gut, Om-cystatin 2 was associated with residual bodies and hemosomes.

Om-cystatins 1 and 2 strongly inhibited papain, cathepsin B and H. Furthermore, Om-cystatin 2 efficiently interacted with cathepsins C, L and S (Grunclová et al., 2006; Salát et al., 2010). The inhibitory activities against papain, cathepsins B and H were also confirmed by the incubation of recombinant Om-cystatins 1 and 2 with midgut tissue followed by measuring the remaining peptidase activity (Grunclová et al., 2006). Om-cystatin 2 was an equally potent inhibitor of cathepsins L and S when compared to SL, but Om-cystatin 2 inhibits much more efficiently cathepsins B, C, and H compared to SL (Salát et al., 2010).

Similar to SL, Om-cystatin 2 was tested for its immunomodulatory characteristics and more specifically for its potential to interact with the host's adaptive immune system (Salát et al., 2010). Cathepsins, such as S, L, and V are involved in the invariant chain degradation of the MHC II complex, and they are responsible for antigen processing by antigen-presenting cells, such as dendritic cells, as well as for antigen presentation in complex with MHC II proteins (Honey and Rudensky, 2003). As part of their physiological functions, dendritic cells – upon their activation by exogenous antigens – produce cytokines and present these foreign antigens to T cells, thus stimulating T-cell proliferation. Om-cystatin 2 was able to suppress TNF- α and IL-12 production in vitro by LPS-activated dendritic cells by 20% and 25%, respectively.

Furthermore, a statistically significant inhibition of the CD4⁺ T-cell proliferation was detected in the presence of Om-cystatin 2. These findings are in line with the immunosuppressive properties of SL on activated dendritic cells (Sá-Nunes et al., 2009). Thus, the recognition of salivary antigens of *O. moubata* is impaired, and tick blood feeding is facilitated. Additionally, the potent inhibition of cathepsins B and H by Om-cystatin 2 may further suppress the recognition of foreign antigens since these cysteine proteases contribute to the degradation of antigens in their processing to be bound to the MHC II complex (Chapman, 2006). This impact on the vertebrate adaptive immunity may further be amplified by the strong inhibition of cathepsin C by Om-cystatin 2. Cathepsin C is involved in the activation of serine proteases in neutrophils, granzymes of cytotoxic T lymphocytes,

and in the physiology of natural killer cells (Pham and Ley, 1999; Adkison et al., 2002). Om-cystatin 2 was also tested for its ability to impair tick feeding on mice. Unlike the SL2 vaccination experiments, no significant differences in the number of ticks fed on mice and the body weight of fed ticks were detected between Om-cystatin 2-immunized mice and the control group (Salát et al., 2010). *O. moubata* nymphs, however, showed significantly increased post-engorgement mortality after feeding on the Om-cystatin 2-immunized mice, and this effect of Om-cystatin 2 on the tick mortality was correlated with the amount of specific anti-Om-cystatin 2 antibodies in mice sera. These antibodies may block the physiological function of Om-cystatin 2 in the tick gut and may contribute to the observed mortality of the engorged ticks (Salát et al., 2010). This is in line with Grunclová et al. (2006) who suggested that both *O. moubata* cystatins are involved in the blood digestion and heme detoxification, while Om-cystatin 2 may target exogenous peptidases in the ingested blood or upon the interaction of ticks with the vertebrate hosts (Salát et al., 2010).

Three more cystatins, two from the tick *O. coriaceus* (NCBI: ACB70345 and ACB70343) and one partial cDNA sequence of a cystatin from *O. parkeri* (NCBI: ABR23498), were discovered in the sialomes of these soft ticks (Francischetti et al., 2008a, 2008b). Both *O. coriaceus* cystatins showed a high amino acid identity to each other (92%), but they are not very similar to other tick cystatins (Fig. 1). The most striking difference between these two *O. coriaceus* cystatins and other type 2 cystatins is the absence of both cystatin loop motifs (lack of QXVXG and PW peptides in their sequence), although these proteins were classified as cystatins. Both cystatins are of secretory nature (see also NCBI BLAST analysis). They are found in the salivary gland proteome of this tick species, and the PW motif is substituted by a PS dipeptide. (Francischetti et al., 2008a). In both *O. coriaceus* cystatins, the third cysteine residue is shifted in the amino acid sequence in comparison with other type 2 cystatins (Fig. 1). These differences in their protein sequence lead to the assumption that the cystatins's inhibitory activity and their function in the tick physiology may be specific and/or unique in comparison to all other biochemically analyzed type 2 tick cystatins.

The cystatin of *O. parkeri* is more similar in its sequence to the known type 2 tick cystatins from hard ticks. It also possesses the conserved loop 1 domain and its four cysteines perfectly align with those in the other cystatin sequences (Fig. 1). This cysteine protease inhibitor also showed an amino acid substitution in loop 2 (PT dipeptide instead of PW), and its secretory properties cannot be directly assessed due to the missing N terminus (a truncated transcript was sequenced), although it seems to belong to the type 2 cystatins based on the characteristics present in the available sequence (Francischetti et al., 2008b).

Of all the functionally analyzed type 2 cystatins that are described in this review, only the cystatins of *O. moubata* significantly inhibited cathepsin B. The decreased activity of the secreted tick cystatins towards the exopeptidase cathepsin B may be explained by the steric hindrance caused by the occluding loop of cathepsin B (Musil et al., 1991). The similarity of the Om-cystatin 2 structure, especially in the N terminus and loop 2, with vertebrate cystatins may explain the broader inhibitory activity of Om-cystatin 2 for several cysteine peptidases in comparison with all the other type 2 cystatins of hard ticks. However, cathepsins such as S and V were not tested for all different secreted cystatins from hard and soft ticks. Accordingly, the emerging picture of a broader spectrum of activity for the soft tick cystatin Om-cystatin 2 towards many different cathepsins has to be further verified by the biochemical characterization of more cystatins coming from soft ticks. Without this information, we cannot conclude whether this is true for all the cystatins of soft tick origin.

Interestingly, all tick cystatins (stefins and type 2 cystatins) described to date lack the legumain/asparaginyl endopeptidase binding site in their protein sequence (SND motif)

which is, for example, typical for human cystatin C (Fig. 1). Legumains from *I. ricinus* (IrAE) and *H. longicornis* (HILgm1 and 2) were discovered in the tick midgut (Sojka et al., 2007; Alim et al., 2007, 2008). IrAE plays a role in the transactivation of other tick peptidases involved in blood digestion. For both *H. longicornis* legumains, a role in blood feeding and a major impact on embryogenesis was observed (Alim et al., 2009). The inefficiency of type 2 tick cystatins to inhibit legumains/asparaginyl endopeptidases suggest that they are rather involved in early processes in the tick physiology such as the interaction with the vertebrate immune response and blood digestion. The regulation of legumain activity in ticks may be carried out by the control of legumain gene expression or by a yet unidentified inhibitor within – or outside – the tick cystatin family.

Conclusions

Worldwide, ticks are important vectors of infectious diseases impacting on human and animal health. Ticks are not only vectors of parasites of livestock that account for diseases such as babesiosis, theileriosis, or anaplasmosis, but they also mediate the transmission of human tick-borne encephalitis and Lyme disease (de Castro, 1997; Alciati et al., 2001). Over the last decades, increases in the tick abundance, such as of *I. ricinus*, the castor bean tick in central Europe, and its infection prevalence with pathogens, such as tick-borne encephalitis virus, have led to an increased risk of human exposure to tick bites and pathogen infection (Daniel et al., 2004; Schwarz et al., 2009).

Tick control currently relies on the use of acaricides, a strategy that can cause serious repercussions such as environmental pollution, contamination of milk and meat products with acaricide residues, and the emergence of acaricide-resistant ticks (de la Fuente and Kocan, 2006). Alternative biocontrol strategies suppress, but do not eliminate the pathogen (Wall, 2007). Targeting ticks by vaccination has also been proposed as an anti-tick strategy, and for this purpose proteins that play an important role in tick feeding success and/or in tick physiology are of high interest. Out of these, proteins that modulate the host immune response are potential vaccine targets as an immunological control strategy.

Until now, the best vaccine (TickGARDTM and GavacTM) to protect cattle from tick infestation is based on a midgut transmembrane protein of *Boophilus microplus* (Bm86) that reduces the number of engorged ticks and tick fecundity by 90% (Willadsen et al., 1989; Willadsen, 2006). However, the direct effect of the vaccine on tick mortality is low and because Bm86 is a ‘concealed’ antigen. It does not naturally boost antibody titers in cattle upon tick exposure. Thus, novel tick antigen candidates for developing anti-tick vaccines are urgently needed in order to control tick infestation on animals as well as to protect humans against tick bites.

In this review, 21 cystatins of ticks were presented. Nine of them were functionally characterized in the reviewed literature, either at the protein level by biochemical assays for their inhibitory properties or by gene silencing experiments that revealed their function in ticks. Some of the identified tick cystatins are not yet biochemically analyzed, and they might be of high interest as potential new candidate antigens for drug or vaccine development. Of these cystatins, HISC-1 of *H. longicornis* or the cystatins of *O. coriaceus* have a unique protein sequence in comparison with other tick cystatins (Francischetti et al., 2008a; Yamaji et al., 2009b). Accordingly, they might display a unique inhibitory profile, or they may have novel, species-specific functions in these ticks. Therefore, further studies on tick cystatins, with special emphasis on their complete biochemical and functional characterization in ticks, are necessary for a better understanding of the contribution of this protein family in the success of ticks as hematophagous arthropods and disease vectors.

All functionally characterized cystatins in this review inhibited cathepsin L. The only exception was Om-cystatin 1, but this cystatin was not tested for its cathepsin L-inhibitory activity. Cathepsin S was also inhibited by SL and Om-cystatin 2 (Kotsyfakis et al., 2006, 2007; Salát et al., 2010). Mammalian cathepsins S, L, and V play an important role in the vertebrate immunity such as their involvement in the antigen presentation processes of dendritic cells and macrophages by regulating the invariant chain cleavages (Zavasnik-Bergant et al., 2006). Changes, for example, in the cathepsin S activity resulted in a redelivery of MHC class II molecules to the lysosomes, and vertebrate cystatin C was shown to regulate the cathepsin S proteolytic activity in dendritic cells (Pierre and Mellman, 1998). Similar to cystatin C, tick cystatins like sialostatins or Om-cystatin 2 are potent immunomodulators, and this similarity suggests that tick protease inhibitors may mimic the function of physiological vertebrate regulators upon pathological challenges such as the feeding of the tick or the transmission of tick-borne pathogens. Accordingly, this mechanism may be generic and apply to other tick salivary effectors that collectively constitute tick saliva, a pluripotent pharmacological cocktail that has an essential role for the success of ticks as disease vectors.

As a consequence of their immunosuppressive characteristics, the neutralization of cystatins (through gene silencing in ticks or vaccination of animals), such as SL2 or the cystatin of *A. americanum*, caused a significant reduction in the ability of ticks to feed on vaccinated hosts and/or impaired tick feeding (Karim et al., 2005; Kotsyfakis et al., 2007, 2008), demonstrating their high importance in successful tick feeding. These cystatins may serve as potent vaccine candidates in order to neutralize the immunosuppressive nature of tick saliva. The discovery of such candidate antigens may bring new perspectives for developing anti-tick vaccines that will protect animals and may also protect humans against tick bites and pathogen transmission. More important, the development of a multicomponent vaccine cocktail that will contain different cystatins (with a hypothetical synergistic effect of these cystatin antigens in this hypothetical cocktail formulation) remains to be tested hopefully in the near future.

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References

- Abrahamson M, Alvarez-Fernandez M, Nathanson CM. Cystatins Biochem. Soc Symp. 2003; 70:179–199.
- Adkison AM, Raptis SZ, Kelley DG, Pham CT. Dipeptidyl peptidase activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. J Clin Invest. 2002; 109:363–371. [PubMed: 11827996]
- Alciati S, Belligni E, Del Colle S, Pugliese A. Human infections tick-transmitted. Panminerva Med. 2001; 43:295–304. [PubMed: 11677426]
- Alim MA, Tsuji N, Miyoshi T, Islam MK, Huang X, Motobu M, Fujisaki K. Characterization of asparaginyl endopeptidase, legumain, induced by blood feeding in the ixodid tick *Haemaphysalis longicornis*. Insect Biochem Mol Biol. 2007; 37:911–922. [PubMed: 17681230]
- Alim MA, Tsuji N, Miyoshi T, Islam MK, Huang X, Hatta T, Fujisaki K. HILgm2, a member of asparaginyl endopeptidases/legumains in the midgut of the ixodid tick *Haemaphysalis longicornis*, is involved in blood-meal digestion. J Insect Physiol. 2008; 54:573–585. [PubMed: 18222467]

- Alim MA, Tsuji N, Miyoshi T, Islam MK, Hatta T, Fujisaki K. Legumains from the hard tick *Haemaphysalis longicornis* play modulatory roles in blood feeding and gut cellular remodelling and impact on embryogenesis. *Int J Parasitol.* 2009; 39:97–107. [PubMed: 18718474]
- Anatriello E, Ribeiro JMC, de Miranda-Santos IKF, Brandão LG, Anderson JM, Valenzuela JG, Maruyama SR, Silva JS, Ferreira BR. An insight into the sialotranscriptome of the brown dog tick, *Rhipicephalus sanguineus*. *BMC Genomics.* 2010; 11:450. [PubMed: 20650005]
- Anderson JM, Sonenshine DE, Valenzuela JG. Exploring the mialome of ticks: an annotated catalogue of midgut transcripts from the hard tick, *Dermacentor variabilis* (Acari: Ixodidae). *BMC Genomics.* 2008; 9:552. [PubMed: 19021911]
- Barrett AJ. Cystatin, the egg white inhibitor of cysteine proteinases. *Methods Enzymol.* 1981; 80:771–778.
- Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem.* 1997; 272:20313–20316. [PubMed: 9252331]
- Björk I, Brieditis I, Raub-Segall E, Pol E, Håkansson K, Abrahamson M. The importance of the second hairpin loop of cystatin C for proteinase binding. Characterization of the interaction of Trp-106 variants of the inhibitor with cysteine proteinases. *Biochemistry.* 1996; 35:10720–10726. [PubMed: 8718861]
- Brown WM, Dziegielewska KM. Friends and relations of the cystatin superfamily –new members and their evolution. *Protein Sci.* 1997; 6:5–12. [PubMed: 9007972]
- Brzin J, Kopitar M, Turk V, Machleidt W. Protein inhibitors of cysteine proteinases. I Isolation and characterization of stefin, a cytosolic protein inhibitor of cysteine proteinases from human polymorphonuclear granulocytes. *Hoppe Seylers Z Physiol Chem.* 1983; 364:1475–1480. [PubMed: 6689311]
- Chapman HA. Endosomal proteases in antigen presentation. *Curr Opin Immunol.* 2006; 18:78–84. [PubMed: 16338127]
- Daniel M, Danielová V, Kříž B, Kott I. An attempt to elucidate the increased incidence of tick-borne encephalitis and its spread to higher altitudes in the Czech Republic. *Int J Med Microbiol.* 2004; 293(Suppl 37):55–62. [PubMed: 15146985]
- de Castro JJ. Sustainable tick and tickborne disease control in livestock improvement in developing countries. *Vet Parasitol.* 1997; 71:77–97. [PubMed: 9261972]
- de la Fuente J, Kocan KM. Strategies for development of vaccines for control of ixodid tick species. *Parasite Immunol.* 2006; 28:275–283. [PubMed: 16842264]
- de la Fuente J, Kocan KM, Blouin EF. Tick vaccines and the transmission of tick-borne pathogens. *Vet Res Commun.* 2007; 31(Suppl 1):85–90. [PubMed: 17682852]
- Dickinson DP. Salivary (SD-type) cystatins: over one billion years in the making – but to what purpose? *Crit Rev Oral Biol Med.* 2002; 13:485–508. [PubMed: 12499242]
- Fossum K, Whitaker JR. Ficin and papain inhibitor from chicken egg white. *Arch Biochem Biophys.* 1968; 125:367–375. [PubMed: 5649527]
- Francischetti IM, Meng Z, Mans BJ, Gudderra N, Hall M, Veenstra TD, Pham VM, Kotsyfakis M, Ribeiro JM. An insight into the salivary transcriptome and proteome of the soft tick and vector of epizootic bovine abortion, *Ornithodoros coriaceus*. *J Proteomics.* 2008a; 71:493–512. [PubMed: 18725333]
- Francischetti IM, Mans BJ, Meng Z, Gudderra N, Veenstra TD, Pham VM, Ribeiro JM. An insight into the sialome of the soft tick, *Ornithodoros parkeri*. *Insect Biochem Mol Biol.* 2008b; 38:1–21. [PubMed: 18070662]
- Gabb HA, Jackson RM, Sternberg MJE. Modelling protein docking using shape complementarity, electrostatics and biochemical information. *J Mol Biol.* 1997; 272:106–120. [PubMed: 9299341]
- Grunclová L, Horn M, Vancová M, Sojka D, Franta Z, Mares M, Kopáček P. Two secreted cystatins of the soft tick *Ornithodoros moubata*: differential expression pattern and inhibitory specificity. *Biol Chem.* 2006; 387:1635–1644. [PubMed: 17132111]
- Grzonka Z, Jankowska E, Kasprzykowski F, Kasprzykowska R, Lankiewicz L, Wiczak W, Wiczerzak E, Ciarkowski J, Drabik P, Janowski R, Kozak M, Jaskólski M, Grubb A. Structural studies of cysteine proteases and their inhibitors. *Acta Biochim Pol.* 2001; 48:1–20. [PubMed: 11440158]

- Halfon S, Ford J, Foster J, Dowling L, Lucian L, Sterling M, Xu Y, Weiss M, Ikeda M, Liggett D, Helm A, Caux C, Lebecques S, Hannum C, Menon S, Mc Clanahan T, Gorman D, Zurawski G. Leukocystatin, a new class II cystatin expressed selectively by hematopoietic cells. *J Biol Chem*. 1998; 273:16400–16408. [PubMed: 9632704]
- Hartmann S, Lucius R. Modulation of host immune responses by nematode cystatins. *Int J Parasitol*. 2003; 33:1291–1302. [PubMed: 13678644]
- Honey K, Rudensky AY. Lysosomal cysteine proteases regulate antigen presentation. *Nat Rev Immunol*. 2003; 3:472–482. [PubMed: 12776207]
- Jacot, MS. PhD thesis. Institute of Zoology, Faculty of Sciences, University of Neuchâtel; Neuchâtel, Switzerland: 2003. Etude des gènes exprimés au cours du repas sanguin dans l'intestin de la tique *Ixodes ricinus*; p. 104
- Kadokura H, Katzen F, Beckwith J. Protein disulfide bond formation in prokaryotes. *Annu Rev Biochem*. 2003; 72:111–135. [PubMed: 12524212]
- Karim S, Miller NJ, Valenzuela JG, Sauer JR, Mather TN. RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding. *Biochem Biophys Res Commun*. 2005; 334:1336–1342. [PubMed: 16040003]
- Keilová H, Tomásek V. Inhibition of cathepsin C by papain inhibitor from chicken egg white and by complex with cathepsin B1. *Coll Czech Chem Commun*. 1975; 40:218–224.
- Kotsyfakis M, Sá-Nunes A, Francischetti IM, Mather TN, Andersen JF, Ribeiro JM. Antiinflammatory and immunosuppressive activity of sialostatin L, a salivary cystatin from the tick *Ixodes scapularis*. *J Biol Chem*. 2006; 281:26298–26307. [PubMed: 16772304]
- Kotsyfakis M, Karim S, Andersen JF, Mather TN, Ribeiro JM. Selective cysteine protease inhibition contributes to blood-feeding success of the tick *Ixodes scapularis*. *J Biol Chem*. 2007; 282:29256–29263. [PubMed: 17698852]
- Kotsyfakis M, Anderson JM, Andersen JF, Calvo E, Francischetti IM, Mather TN, Valenzuela JG, Ribeiro JM. Cutting edge: Immunity against a “silent” salivary antigen of the Lyme vector *Ixodes scapularis* impairs its ability to feed. *J Immunol*. 2008; 181:5209–5212. [PubMed: 18832673]
- Kotsyfakis M, Horka H, Salat J, Andersen JF. The crystal structures of two salivary cystatins from the tick *Ixodes scapularis* and the effect of these inhibitors on the establishment of *Borrelia burgdorferi* infection in a murine model. *Mol Microbiol*. 2010; 77:456–470. [PubMed: 20545851]
- Kozakov D, Hall DR, Beglov D, Brenke R, Comeau SR, Shen Y, Li K, Zheng J, Vakili P, Paschalidis IC, Vajda S. Achieving reliability and high accuracy in automated protein docking: Cluspro, PIPER, SDU, and stability analysis in CAPRI rounds 13–19. *Proteins: Struct Funct Bioinf*. 2010; 78:3124–3130.
- Lefebvre C, Cocquerelle C, Vandenbulcke F, Hot D, Huot L, Lemoine Y, Salzert M. Transcriptomic analysis in the leech *Theromyzon tessulatum*: involvement of cystatin B in innate immunity. *Biochem J*. 2004; 380:617–625. [PubMed: 15089746]
- Lenar i , B.; Ritonja, A.; Sali, A.; Kotnik, M.; Turk, V.; Machleidt, W. Cysteine Proteinases and their Inhibitors. Turk, V., editor. Walter de Gruyter; Berlin: 1986. p. 473–487.
- Lima CA, Sasaki SD, Tanaka AS. Bmcystatin, a cysteine proteinase inhibitor characterized from the tick *Boophilus microplus*. *Biochem Biophys Res Commun*. 2006; 347:44–50. [PubMed: 16806070]
- Lombardi G, Burzyn D, Mundiñano J, Berguer P, Bekinschtein P, Costa H, Castillo LF, Goldman A, Meiss R, Piazzon I, Nepomnaschy I. Cathepsin-L influences the expression of extracellular matrix in lymphoid organs and plays a role in the regulation of thymic output and of peripheral T cell number. *J Immunol*. 2005; 174:7022–7032. [PubMed: 15905545]
- Lustigman S, Brotman B, Huima T, Prince AM. Characterization of an *Onchocerca volvulus* cDNA clone encoding a genus specific antigen present in infective larvae and adult worms. *Mol Biochem Parasitol*. 1991; 45:65–75. [PubMed: 2052041]
- Lustigman S, Brotman B, Huima T, Prince AM, McKerrow JH. Molecular cloning and characterization of onchocystatin, a cysteine proteinase inhibitor of *Onchocerca volvulus*. *J Biol Chem*. 1992; 267:17339–17346. [PubMed: 1512269]
- Machleidt W, Borchart U, Fritz H, Brzin J, Ritonja A, Turk V. Protein inhibitors of cysteine proteinases. II Primary structure of stefin, a cytosolic protein inhibitor of cysteine proteinases from

- human polymorphonuclear granulocytes. Hoppe Seylers Z Physiol Chem. 1983; 364:1481–1486. [PubMed: 6689312]
- Morales FC, Furtado DR, Rumjanek FD. The N-terminus moiety of the cystatin SmCys from *Schistosoma mansoni* regulates its inhibitory activity in vitro and in vivo. Mol Biochem Parasitol. 2004; 134:65–73. [PubMed: 14747144]
- Musil D, Zucic D, Turk D, Engh RA, Mayr I, Huber R, Popovic T, Turk V, Towatari T, Katunuma N, Bode W. The refined 2.15 Å X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity. EMBO J. 1991; 10:2321–2330. [PubMed: 1868826]
- Müller-Esterl W, Fritz H, Machleidt W, Ritonja A, Brzin J, Kotnik M, Turk V, Kellermann J, Lottspeich F. Human plasma kininogens are identical with alpha-cysteine proteinase inhibitors. Evidence from immunological, enzymological and sequence data. FEBS Lett. 1985; 182:310–314. [PubMed: 2579850]
- Ohkubo I, Kurachi K, Takasawa T, Shiokawa H, Sasaki M. Isolation of a human cDNA for alpha 2-thiol proteinase inhibitor and its identity with low molecular weight kininogen. Biochemistry. 1984; 23:5691–5697. [PubMed: 6441591]
- Pham CT, Ley TJ. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. Proc Natl Acad Sci USA. 1999; 96:8627–8632. [PubMed: 10411926]
- Pierre P, Mellman I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. Cell. 1998; 93:1135–1145. [PubMed: 9657147]
- Rawlings ND, Barrett AJ. Evolution of proteins of the cystatin superfamily. J Mol Evol. 1990; 30:60–71. [PubMed: 2107324]
- Rawlings ND, Morton FR, Barrett AJ. MEROPS: the peptidase database. Nucleic Acids Res. 2006; 34:D270–272. [PubMed: 16381862]
- Rawlings ND, Barrett AJ, Bateman A. MEROPS: the peptidase database. Nucleic Acids Res. 2010; 38:D227–233. [PubMed: 19892822]
- Reddy VY, Zhang QY, Weiss SJ. Pericellular mobilization of the tissue-destructive cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived macrophages. Proc Natl Acad Sci USA. 1995; 92:3849–3853. [PubMed: 7731994]
- Ribeiro JM, Alarcon-Chaidez F, Francischetti IM, Mans BJ, Mather TN, Valenzuela JG, Wikel SK. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. Insect Biochem Mol Biol. 2006; 36:111–129. [PubMed: 16431279]
- Ribeiro JM, Anderson JM, Manoukis NC, Meng Z, Francischetti IM. A further insight into the sialome of the tropical bont tick, *Amblyomma variegatum*. BMC Genomics. 2011; 12:136. [PubMed: 21362191]
- Ritonja A, Machleidt W, Barrett AJ. Amino acid sequence of the intracellular cysteine proteinase inhibitor cystatin B from human liver. Biochem Biophys Res Commun. 1985; 131:1187–1192. [PubMed: 3902020]
- Sá-Nunes A, Bafica A, Antonelli LR, Choi EY, Francischetti IM, Andersen JF, Shi GP, Chavakis T, Ribeiro JM, Kotsyfakis M. The immunomodulatory action of sialostatin L on dendritic cells reveals its potential to interfere with autoimmunity. J Immunol. 2009; 182:7422–7429. [PubMed: 19494265]
- Salát J, Paesen GC, Rezáková P, Kotsyfakis M, Kovárová Z, Sanda M, Majtán J, Grunclová L, Horká H, Andersen JF, Brynda J, Horn M, Nunn MA, Kopáček P, Kopecký J, Mares M. Crystal structure and functional characterization of an immunomodulatory salivary cystatin from the soft tick *Ornithodoros moubata*. Biochem J. 2010; 429:103–112. [PubMed: 20545626]
- Scharfstein J, Schmitz V, Svensjö E, Granato A, Monteiro AC. Kininogens coordinate adaptive immunity through the proteolytic release of bradykinin, an endogenous danger signal driving dendritic cell maturation. Scand J Immunol. 2007; 66:128–136. [PubMed: 17635790]
- Schönemeyer A, Lucius R, Sonnenburg B, Brattig N, Sabat R, Schilling K, Bradley J, Hartmann S. Modulation of human T cell responses and macrophage functions by onchocystatin, a secreted protein of the filarial nematode *Onchocerca volvulus*. J Immunol. 2001; 167:3207–3215. [PubMed: 11544307]
- Schierack P, Lucius R, Sonnenburg B, Schilling K, Hartmann S. Parasite-specific immunomodulatory functions of filarial cystatin. Infect Immun. 2003; 71:2422–2429. [PubMed: 12704112]

- Schwarz A, Maier WA, Kistemann T, Kampen H. Analysis of the distribution of the tick *Ixodes ricinus* L. (Acari: Ixodidae) in a nature reserve of western Germany using Geographic Information Systems. *Int J Hyg Environ Health*. 2009; 212:87–96. [PubMed: 18262840]
- Sen LC, Whitaker JR. Some properties of a ficin-papain inhibitor from avian egg white. *Arch Biochem Biophys*. 1973; 158:623–632. [PubMed: 4782525]
- Sojka D, Hajdusek O, Dvorák J, Sajid M, Franta Z, Schneider EL, Craik CS, Vancová M, Buresová V, Bogyo M, Sexton KB, McKerrow JH, Caffrey CR, Kopáček P. IrAE: an asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. *Int J Parasitol*. 2007; 37:713–724. [PubMed: 17336985]
- Sotiropoulou G, Anisonowicz A, Sager R. Identification, cloning, and characterization of cystatin M, a novel cysteine proteinase inhibitor, down-regulated in breast cancer. *J Biol Chem*. 1997; 272:903–910. [PubMed: 8995380]
- Stubbs MT, Laber B, Bode W, Huber R, Jerala R, Lenarcic B, Turk V. The refined 2.4 Å X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. *EMBO J*. 1990; 9:1939–1947. [PubMed: 2347312]
- Tarasuk M, Vichasri Grams S, Viyanant V, Grams R. Type I cystatin (stefin) is a major component of *Fasciola gigantica* excretion/secretion product. *Mol Biochem Parasitol*. 2009; 167:60–71. [PubMed: 19416741]
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994; 22:4673–4680. [PubMed: 7984417]
- Tsuji N, Miyoshi T, Battsetseg B, Matsuo T, Xuan X, Fujisaki K. A cysteine protease is critical for *Babesia* spp. transmission in *Haemaphysalis* ticks. *PLoS Pathog*. 2008; 4:e1000062. [PubMed: 18483546]
- Turk V, Stoka V, Turk D. Cystatins: Biochemical and structural properties, and medical relevance. *Front Biosci*. 2008; 13:5406–5420. [PubMed: 18508595]
- Valenzuela JG, Francischetti IM, Pham VM, Garfield MK, Mather TN, Ribeiro JMC. Exploring the sialome of the tick *Ixodes scapularis*. *J Exp Biol*. 2002; 205:2843–2864. [PubMed: 12177149]
- Vray B, Hartmann S, Hoebeke J. Immunomodulatory properties of cystatins. *Cell Mol Life Sci*. 2002; 59:1503–1512. [PubMed: 12440772]
- Wall R. Ectoparasites: future challenges in a changing world. *Vet Parasitol*. 2007; 148:62–74. [PubMed: 17560724]
- Wasilewski MM, Lim KC, Phillips J, McKerrow JH. Cysteine protease inhibitors block schistosome hemoglobin degradation in vitro and decrease worm burden and egg production in vivo. *Mol Biochem Parasitol*. 1996; 81:179–189. [PubMed: 8898333]
- Willadsen P, Riding GA, McKenna RV, Kemp DH, Tellam RL, Nielsen JN, Lahnstein J, Cobon GS, Gough JM. Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*. *J Immunol*. 1989; 143:1346–1351. [PubMed: 2745982]
- Willadsen P. Tick control: thoughts on a research agenda. *Vet Parasitol*. 2006; 138:161–168. [PubMed: 16497440]
- Wille A, Gerber A, Heimbürg A, Reisenauer A, Peters C, Saftig P, Reinheckel T, Welte T, Bühling F. Cathepsin L is involved in cathepsin D processing and regulation of apoptosis in A549 human lung epithelial cells. *Biol Chem*. 2004; 385:665–670. [PubMed: 15318816]
- Yamaji K, Tsuji N, Miyoshi T, Islam MK, Hatta T, Alim MA, Anisuzzaman M, Takenaka A, Fujisaki K. Hemoglobinase activity of a cysteine protease from the ixodid tick *Haemaphysalis longicornis*. *Parasitol Int*. 2009a; 58:232–237. [PubMed: 19446040]
- Yamaji K, Tsuji N, Miyoshi T, Islam MK, Hatta T, Alim MA, Anisuzzaman M, Kushibiki S, Fujisaki K. A salivary cystatin, HISC-1, from the ixodid tick *Haemaphysalis longicornis* play roles in the blood-feeding processes. *Parasitol Res*. 2009b; 106:61–68. [PubMed: 19779741]
- Yamaji K, Tsuji N, Miyoshi T, Hatta T, Alim MA, Anisuzzaman M, Kushibiki S, Fujisaki K. Hlcyst-1 and Hlcyst-2 are potential inhibitors of HICPL-A in the midgut of the ixodid tick *Haemaphysalis longicornis*. *J Vet Med Sci*. 2010; 72:599–604. [PubMed: 20103991]
- Zavasnik-Bergant T, Turk B. Cysteine cathepsins in the immune response. *Tissue Antigens*. 2006; 67:349–355. [PubMed: 16671941]

- Zavasnik-Bergant T. Cystatin protease inhibitors and immune functions. *Front Biosci.* 2008; 13:4625–4637. [PubMed: 18508534]
- Zhou J, Ueda M, Umehiya R, Battsetseg B, Boldbaatar D, Xuan X, Fujisaki K. A secreted cystatin from the tick *Haemaphysalis longicornis* and its distinct expression patterns in relation to innate immunity. *Insect Biochem Mol Biol.* 2006; 36:527–535. [PubMed: 16835018]
- Zhou J, Liao M, Ueda M, Gong H, Xuan X, Fujisaki K. Characterization of an intracellular cystatin homolog from the tick *Haemaphysalis longicornis*. *Vet Parasitol.* 2009; 160:180–183. [PubMed: 19091470]
- Zhou J, Liao M, Gong H, Xuan X, Fujisaki K. Characterization of Hlcyst-3 as a member of cystatins from the tick *Haemaphysalis longicornis*. *Exp Appl Acarol.* 2010; 51:327–333. [PubMed: 20107871]

Fig. 1.

Alignment of all known tick cystatins. All type 1 and 2 tick cystatins from the literature were aligned using the program CLustalW (Thompson et al., 1994), and only the mature proteins (without the signal peptide) are presented. The N-terminal glycine (depicted as N on top of the alignment), loop 1 with the QXVXG segment (depicted as L1 on top of the alignment), and loop 2 with the PW peptide, if present, (depicted as L2 on top of the alignment) of the cystatin domain are boxed. Possible disulfide bridges which are formed between cysteine residues of secreted cystatins (type 2) are marked with (S-S) in the bottom of the alignment, and the presumed legumain (asparaginyl endopeptidase) binding site (SND motif) of cystatin C is boxed and marked with an asterisk on top of the alignment. The conserved cystatin domains and other conserved amino acid residues of the cystatins are highlighted in black; identical residues are marked in gray. The following type 1 cystatins are presented: Iscap: *I. scapularis* cystatin (AAY66864); Bmcystatin of *R. microplus* (ABG36931); RsangInt: intracellular cystatin of *R. sanguineus* (ACX53850); Hlcyst-1 of *H. longicornis* (ABZ89553); DvM602 of *D. variabilis* (ACF35512); CystA, B: human cystatins A (P01040) and B (P04080). Type 2 cystatins are: Cyst C: human cystatin C (P01034); Sialo L, L2: Sialostin L (AAM93646) and L2 of *I. scapularis* (AAY66685); Aamer: cystatin of *A. americanum*; Irici: cystatin of *I. ricinus* (CAD68002); Avari: cystatin of *A. variabilis* (DAA34288); RsangSec1 (ACX53922), RsangSec2 (ACX53862): secreted cystatins of *R. sanguineus*, DvM334: cystatin of *D. variabilis* (ACF35514); Hlcyst-2 (ABC94582), 3 (ABZ89554), HlSC-1 of *H. longicornis* (BAI59105); Om-cystatin 1 (AAS01021), 2 of *O. moubata* (AAS55948); Opark: cystatin of *O. parkeri* (ABR23498); Ocori 1 (ACB70345), 2 (ACB70343): cystatins of *O. coriaceus*.

Fig. 2.

Sialostatin L2 docking with Cathepsin L. A ClusPro docking (Kozakov et al., 2010) was carried out for Sialostatin L2 with cathepsin L using protein monomers from the RCSB Protein Data Bank (PDB). To test the efficiency of ClusPro docking stefin A (PDB code: 1DVC) and Chagasin (2NNR), a cysteine protease inhibitor of *Trypanosoma cruzi*, were initially docked with cathepsin L (1CS8). These specific cystatins were chosen because both have complex structures with cathepsin L in the PDB (3KSE for stefin A and 2NQD for Chagasin), thus allowing us to test for any structural deviation after docking. For both of these docked monomers the top pose from the ClusPro server produced a docking with a root mean square deviation (RMSD) of ~ 8 Å when superimposed with its respective complex structure from the PDB. According to Gabb et al. (1997), an RMSD ~ 10 Å is indicative of a successful docking pose. (A) Ribbon representation of the top ClusPro docking pose for Sialostatin L2 (red; 3LH4) and cathepsin L (blue; 1CS8). The diagram depicts the characteristics of secreted type 2 cystatins, namely the two loops (depicted as L1 and L2 in the figure) of the conserved cystatin domain, the N terminus (depicted as N-Ter in the figure) of Sialostatin L2, and the disulfide bridges (yellow; α -carbons are in pink). (B) A detailed representation of Sialostatin L2 interacting within the binding site of cathepsin L (grey spheres). Sialostatin L2 side chains for loops 1 and 2 (red) are in stick format representing the conserved QXVXG motif (here QTVAG, position 52–56 in the protein sequence) for loop 1 and the NL dipeptide (position 99–100) for loop 2 that corresponds to the PW dipeptide in other type 2 cystatins.

Table 1
Overview of all known tick cystatins: Biochemical characteristics and their physiological function in ticks.

Tick species	Name of inhibitor	Type ^a	MW (kDa)	pI [*]	NCBI number	Tissue specificity	Cysteine protease inhibition	Cystatin function	References
<i>A.</i>	Cyst	2	15	5.	–	M, SG (s)	–	Regulation of host	Karim et al.
<i>A. variegatum</i>	Cystatin	2	10.5 ¹	6.9 ¹	DAA34288	–	–	–	Ribeiro et al. (2011)
<i>D. variabilis</i>	Dv M602	1	11.1 [*]	5.8 [*]	ACF35512	M (s)	–	–	Anderson et al. (2008)
	Dv M334	2	9.7 ^{*1}	4.5 ^{*1}	ACF35514	M (s)	–	–	Anderson et al. (2008)
<i>H. longicornis</i>	Hlcyst-1	1	11.0	5.5	ABZ89553	M (s)	Papain cath B, H, L HICPL-A	Regulation of hemoglobin digestion (r)	Zhou et al. (2006, 2009); Yamaji et al. (2010)
	Hlcyst-2	2	12.9	8.5	ABC94582	M (s, p) H, SG, O, F (p)	Papain cath L, H HICP L-A	Regulation of haemoglobin digestion and tick innate immunity (r)	Zhou et al. (2006); Yamaji et al. (2009, 2010)
	Hlcyst-3	2	11.0	4.5	ABZ89554	M (s, p); H, SG, O, F (p)	Papain cath L	Role in the tick midgut physiology and/or tick innate immunity (r)	Zhou et al. (2006, 2010)
	HIS C-1	2	12.4	9.8	BAI59105	SG (s, p) M (p)	Papain cath L	Role in blood feeding (r)	Yamaji et al. (2009, 2010)
<i>I. ricinus</i>	Cystatin	2	14.0	8.4 [*]	CAD68002	M (p, a)	–	–	Jacob (2003)
<i>I. scapularis</i>	Cystatin	1	9.0 [*]	7.0 [*]	AAY66864	SG (s)	–	–	Lima et al. (2006)
	Sialostat in L	2	11.9	4.9	AAM93646	SG (s, p, a) M (p)	Papain cath C, L, V, S	Anti-inflammatory and immunomodulatory properties (t)	Valenzuela et al. (2002); Kotsyfakis et al. (2006, 2007, 2010); Sá-Nunes et al. (2009)
	Sialostat in L2	2	12.5	5.3 [*]	AAY66685	SG (s, p) M (p)	Papain cath L, V	Immunosuppressive properties aid tick feeding and pathogen transmission (t)	Kotsyfakis et al. (2007, 2008, 2010); Ribeiro et al. (2006)
<i>O. moubata</i>	Om-cystatin 1	2	12.4	7.8	AAS01021	M (s, p, a)	Papain cath B, H	Role in the blood digestion and haem detoxification (r)	Grunclová et al. (2006)
	Om-cystatin 2	2	12.2	5.4	AAS55948	M (s, p, a) SG(p, a); O (p); Mal (p)	Papain cath B, C, H, L, S	Role in the blood digestion, haem detoxification and modulation of the host immune response (t)	Grunclová et al. (2006); Salát et al. (2010)
<i>O. coriaceus</i>	Cystatin	2	13.0 [*]	10.2 [*]	ACB70345	SG (s)	–	–	Francischetti et al. (2008a)

Tick species	Name of inhibitor	Type ^a	MW (kDa) *	pI [*]	NCBI number	Tissue specificity	Cysteine protease inhibition	Cystatin function	References
<i>O. parkeri</i>	Cystatin	2	12.9*	9.7*	ACB70343	SG (s)	–	–	Francischetti et al. (2008a)
	Cystatin	2	12.9* ¹	9.8* ¹	ABR23498	SG (s)	–	–	Francischetti et al. (2008b)
<i>R. microplus</i>	Bmcystatin	1	11.0	5.7	ABG36931	F (s, a); O (p, a); SG (a)	cath L VTD CE	Regulation of tick embryogenesis (r)	Lima et al. (2006)
<i>R. sanguineus</i>	Cystatin	1	9.5*	6.3*	ACX53850	SG (s)	–	–	Anatriello et al. (2010)
	Cystatin	2	15.5*	9.5*	ACX53922	SG (s)	–	–	Anatriello et al. (2010)
	Cystatin	2	13.1*	5.0*	ACX53862	SG (s)	–	–	Anatriello et al. (2010)

^aType 1 or 2 cystatins correspond to clan IH inhibitors of the I25A or I25B families, respectively, according to the MEROPS peptidase database nomenclature.

Molecular weights (MW) and isoelectric points (pI) for mature proteins are presented. Theoretically calculated MWs and/or pIs using the ExPASy Bioinformatics Resource Portal are additionally marked with an asterisk (); all other values derive from the respective references. Calculations for only partial protein sequences are indicated (†).

SG, salivary glands; M, midgut; H, hemocytes; O, ovary; F, fat body; Mal, Malpighian tubules; cath, cathepsin.

s, p, a: Cystatins were either detected by sequencing (s), PCR (p), or with polyclonal antibodies in immunoassays (a).

HICPL-A: cathepsin L-like cysteine protease of *H. longicornis*.

VTDCE: Vitellin-degrading cysteine endopeptidase of *R. microplus*.

t, r: The biological function for each inhibitor was either tested (t) experimentally or their function was assumed by the referenced authors (r).

In some cases, only a few cathepsins were tested in biochemical assays for cystatin inhibition.