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REVIEW

Cysteine proteases in protozoan parasites

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Abstract

Cysteine proteases (CPs) play key roles in the pathogenesis of protozoan parasites, including cell/tissue penetration, hydrolysis of host or parasite proteins, autophagy, and evasion or modulation of the host immune response, making them attractive chemotherapeutic and vaccine targets. This review highlights current knowledge on clan CA cysteine proteases, the best-characterized group of cysteine proteases, from 7 protozoan organisms causing human diseases with significant impact: *Entamoeba histolytica*, *Leishmania* species (sp.), *Trypanosoma brucei*, *T. cruzi*, *Cryptosporidium* sp., *Plasmodium* sp., and *Toxoplasma gondii*. Clan CA proteases from three organisms (*T. brucei*, *T. cruzi*, and *Plasmodium* sp.) are well characterized as druggable targets based on in vitro and in vivo models. A number of candidate inhibitors are under development. CPs from these organisms and from other protozoan parasites should be further characterized to improve our understanding of their biological functions and identify novel targets for chemotherapy.

Introduction

Proteases are enzymes that catalyze the hydrolysis of peptide bonds and are important in a number of biological activities, including digestion of peptides, activation of other enzymes, modulation of the immune system, participation in the cell cycle, and differentiation and autophagy. There are at least 6 classes of proteases classified according to the nucleophilic group responsible for the first step in the proteolysis: serine, cysteine, metallo, aspartate, glutamate, and threonine proteases. Cysteine proteases (CPs) are categorized into 72 families, but not all are represented in protozoan parasites [1]. The most abundant and well characterized CPs in these organisms are the clan CA papain-family enzymes, named after an abundant protease present in papaya fruit. We selected 7 protozoan organisms with medical relevance for review of the current knowledge regarding their clan CA CPs. Table 1 summarizes well-characterized clan CA CPs from these organisms, and Table 2 lists some well-studied inhibitors.

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Despite the essentiality of CPs for parasite survival, there are, as yet, no approved drugs targeting this group of enzymes. The most advanced drug in development against a parasite CP is the vinyl sulfone inhibitor K11777, with in vivo efficacy in an animal model of Chagas disease [2, 3]; K11777 is currently moving forward to clinical trials.

Entamoeba histolytica

E. histolytica causes amebiasis and is responsible for about 70,000 deaths per year [4, 5]. The parasite infects the intestinal tract and can cause diarrhea, colitis, and peritonitis. Extraintestinal amebiasis can also occur, commonly leading to amoebic liver abscesses. *E. histolytica* CPs are encoded by approximately 50 genes and are responsible for epithelial barrier penetration and degradation of host extracellular matrix components. Only four proteases, EhCP1, EhCP2, EhCP5, and EhCP7, are highly expressed by *E. histolytica* in culture and have identified cellular localizations. EhCP1 is present in intracellular vesicles, EhCP2 localizes to the internal and external cell membrane, and EhCP5 is on the cell surface. Invasive *E. histolytica* clinical isolates have 10×–1,000× more CP activity in culture than noninvasive *E. dispar* isolates, suggesting a role for CPs in parasite virulence and invasion [6].

Secreted *E. histolytica* CPs cleave the colonic mucus layer and overcome mucosal host defenses in the gut [7]. Villin and tight junction proteolysis are also mediated by these CPs [8]. EhCP1, EhCP2, and EhCP5 have been shown to degrade extracellular matrix components, such as fibronectin, laminin, and collagen [9]. Transcriptional studies identified increased expression of EhCP1 and EhCP2 when *E. histolytica* interacted with human collagen and mucin [10]. Those results corroborate the key role of these CPs in parasite invasion of host tissues.

E. histolytica CPs are also hypothesized to interfere with both innate and adaptive host immune responses. They degrade human IgA, the most abundant immunoglobulin defense at the mucosal surface [11]. Extracellular CPs disrupt the heavy chain of IgG, and this is considered to be a key strategy for *E. histolytica* survival during tissue invasion [12]. *E. histolytica* CPs activate the alternative pathway of the host complement system and generate hemolytically active C3b, but extracellular CPs circumvent host immunity by degrading and inactivating anaphylatoxins C3a and C5a [13]. *E. histolytica* CPs modulate cell-mediated immunity as well. Extracellular CPs degrade pro–IL-1β that is released by damaged intestinal epithelial cells to form the active mature proinflammatory cytokine IL-1β [14]. On the other hand, *E. histolytica* CPs cleave both pro–and mature IL-18 [15], and the degradation of IL-18 may contribute to either parasite or host survival. A recent study demonstrated the function of EhCP1, EhCP4, and EhCP5 in the intercellular junction of *E. histolytica* and macrophages to modulate the host cell cytoskeleton and trigger subsequent IL-1β secretion, detailing the cascade of molecular events triggered by the initial interaction of *E. histolytica* with macrophages [16]. That study suggests new roles for EhCP1 and EhCP4 in inducing a proinflammatory response by host macrophages upon cellular contact. EhCPs may also contribute to parasite encystation–excystation [17] and in nutrient acquisition through erythrophagocytosis [18], both essential to parasite infectivity and survival.

The vinyl sulfone inhibitors K11777 and WRR483 were tested against recombinant EhCP1, and both demonstrated potent inhibitory activity, significantly reducing parasite invasion of human colonic xenografts [19]. Further studies should be carried out to develop an antiamebiasis drug targeting CPs.

Leishmania sp.

Leishmaniasis is a spectrum of diseases, including skin ulcers; degradation of nose and palate mucosal tissue; and liver, spleen, and bone marrow infiltration. This latter visceral form of the

Table 1. CPs of protozoan parasites.

Protozoan parasite	Enzymes	EuPathDB entry	Proposed function	Reference
Entamoeba histolytica	EhCP1	EHI_074180	Invasion, virulence factors, induces macrophage proinflammatory response, tight junction proteolysis, degradation of ECM (fibronectin, laminin, collagen)	[8, 10, 16, 18]
	EhCP2	EHI_033710	Invasion, virulence factors, induces macrophage proinflammatory response, tight junction proteolysis, degradation of ECM (fibronectin, laminin, collagen)	[6–8, 10, 16, 18]
	EhCP5	EHI_168240	Invasion, virulence factors, induces macrophage proinflammatory response, degradation of ECM (fibronectin, laminin, collagen), degradation of IgA, IgG, pro–IL-1β, pro–and mature IL-18 (immunoevasion), inactivation of anaphylatoxins	[10–15]
	EhCP7	EHI_039610	Invasion, virulence factors, induces macrophage proinflammatory response	[16, 18]
	EhCP (unspecified)	unspecified	Virulence factor, host defense evasion	[6–8]
	EhCP (unspecified)	unspecified	Parasite encystation–excystation	[17]
Leishmania sp.	CPA, CPB1, CPB2	LmxM.19.1420, LmjF.19.1420, LinJ.19.1460; LmxM.07.0550, LmjF.08.1010;	Virulence factor; immunomodulators; parasite metabolism modulator	[26, 33, 35, 37, 149]
	CPC	LmxM.08_29.0820, LmjF.29.0820, LinJ.29.0860	In vitro virulence factor; role in parasite cell death	[42]
	Atg4	LmjF.30.0270, LinJ.30.0270	Autophagy (differentiation and virulence)	[41, 150]
	otubain	LmxM.17.1400, LmjF.17.1400, LinJ.17.1520	Deubiquitination, proinflammatory activity	[42]
Trypanosoma brucei	TbCatL (brucipain, rhodesain)	Tb927.6.960-Tb927.6.1060	CatL-like activity, virulence factor, promotes crossing of the BBB, host immune evasion	[51, 54, 151]
	TbCatB	Tb427.06.560	Virulence factor, iron acquisition	[59–61, 152]
T. cruzi	Cruzain (GP57/51, TcCatL)	TCDM_05847-t26_1	Virulence factor, evasion of immune system, invasion of host cells, parasite differentiation	[2, 3, 73]
	Calpain-like (TcCatB)	TcCLB.506227.130, TCDM_01392	Signaling transduction, metacyclogenesis	[75]
	Atg4	TCDM_05817-t26_1, TCDM_06287-t26_1	Parasitic autophagy important in differentiation and virulence	[76, 153]
Cryptosporidium sp.	Cryptopain (CatL-like, CpaCATL-1)	cubi_02618-t31_1, cgd3_680, cgd4_2110, cgd6_4880, cgd7_2850	Host cell invasion	[89, 90]
	CpOTU (otubain)	cgd6_2750	Ubiquitin pathway; peak in the oocyst stage	[93]
Plasmodium sp.	Falcipain-1	PF3D7_1458000.1	Oocyst production	[110]
	SERA family. Atypical CP and dipeptidyl aminopeptidase 3.	PF3D7_0207500	Invasion of hepatocytes by sporozoites	[103, 106]
	Falcipain-2, falcipain-3, dipeptidyl aminopeptidase 1	PF3D7_1115700.1, PF3D7_1115400.1	Hemoglobin hydrolysis	[101, 122]
	PfOTU	PF3D7_1031400	Apicoplast development and homeostasis	[115]
Toxoplasma sp.	Single copy gene CatB (TgCPB)	TGME49_249670	Cell invasion and replication	[140, 142]
	Single copy gene CatL (TgCPL)	TGME49_321530	Host cell invasion, digestion of cytosolic proteins	[134, 141]
	TgCP1 and TgCP2 (CatC-like), exopeptidases	TGME49_289620, TGME49_276130	Expressed in tachyzoites, degrade peptides	[137]

Abbreviations: BBB, blood–brain barrier; CP, cysteine proteases; ECM, extracellular matrix; EuPathDB, Eukaryotic Pathogen Database; sp., species.

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Table 2. CP inhibitors.

Inhibitor	Targeted enzyme	Organisms/disease	References
K11777	Cruzain, TcCatL, cryptopains, TgCPL, TgCPB, EhCP1, LtCP	*Trypanosoma cruzi*, *T. brucei*, *Cryptosporidium parvum*, *Toxoplasma gondii*, *Entamoeba histolytica*, *Leishmania tropica*	[2, 19, 60, 91, 149]
WRR-483	Cruzain, EhCP1	*T. cruzi*, *E. histolytica*	[19, 82, 154–156]
Cz007, Cz008	Cruzain	*T. cruzi*	[81]
Gallinamide A	Falcipain-2, falcipain-3	*Plasmodium falciparum*	[128]
Symplostatin 4	Falcipain	*P. falciparum*	[157]
Tetrafluorophenoxymethyl ketone	Cruzain	*T. cruzi*	[158]
Odanacatib	Human cathepsin K	osteoporosis	[159]
Balicatib	Human cathepsin K	osteoporosis	[160]
Emricasan	Human caspase-8	Liver protection, leukemia	[161]
SAR114137	Human cathepsin S	Chronic pain	https://ncats.nih.gov/files/SAR114137.pdf
RWJ-445380	Human cathepsin S	Rheumatoid arthritis	https://ncats.nih.gov/files/RWJ-445380.pdf

Abbreviation: CP, cysteine protease.

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disease is the most lethal, killing over 60,000 people per year [20]. The factors determining clinical manifestations are not fully understood; these are strongly influenced by parasite strain or species and by immunological factors [21]. *Leishmania* express a broad range of CPs, the best characterized of which are CPA, CPB, and CPC (CPs A, B, and C), all part of clan CA, family C1.

CPA and CPB are cathepsin L-like and likely show some functional redundancy [22], while CPC is cathepsin B-like. Cathepsin B-like and cathepsin L-like enzymes have key structural differences associated with divergent substrate binding and proteolytic mechanisms. In cathepsin L, binding of the peptide substrates spans the entire channel between the two domains of the protein, leading to endopeptidolytic activity. In contrast, cathepsin B has an additional occlusion loop that restricts substrate binding under low pH, leading to carboxypeptidase activity; this loop is displaced at high pH, at which point cathepsin B displays endopeptidase activity [23, 24].

CPB genes are arranged in tandem arrays [25], with 19 copies in *L. mexicana*, 8 in *L. major* [26], and 5 in *L. chagasi* [27], while CPA and CPC are single copy [28, 29]. CP gene expression is stage regulated; most are expressed at higher levels in the mammalian amastigote stage than in the insect promastigote stage [25, 29]. However, CPB1 and CPB2 are expressed at higher levels in metacyclic promastigotes [30] and CPC in procyclic promastigotes [28]. Consistent with elevated CP expression in the amastigote stage, *Leishmania* CPs play key roles in the interactions between *Leishmania* and its mammalian host. CPA-knockdown or knockout *L. chagasi* and *L. infantum* show decreased in vitro [31] and in vivo infectivity [32]. Likewise, CPB-knockout *L. mexicana* displayed impaired macrophage infectivity and delayed lesion progression [33]. Multiple CPB copies are required for significant restoration of virulence [34]. CPB modulates host responses to *L. mexicana* by down-regulating protective Th1 immune responses, and in particular IFN-γ production, via degradation of the transcription factor NF-κB and subsequent inhibition of IL-12 production by infected macrophages [35]. *L. mexicana* CPs also cleave JNK and ERK MAP kinases. Both kinases negatively regulate IL-12 production, and therefore the protozoan cysteine protease CPB can alter host macrophage signaling by increasing IL-12 transcription [35]. *L. amazonensis* likewise inhibits antigen presentation via CP-mediated degradation of MHC class II molecules [36]. CPB also modulates levels of parasite proteins, including gp63 [37], a major virulence factor in *Leishmania*. Episomal expression

of gp63 in CPB-deficient parasites was sufficient to restore virulence to wild-type levels [37]. Several of the effects attributed to CPB via knockout studies may therefore occur via modulation of other parasite proteins rather than through direct cleavage by CPB. CPC, in contrast, is involved in *Leishmania* cell death [38] and secretome remodeling [39]; knockouts were attenuated in macrophage infection in vitro but were nevertheless able to cause lesions in vivo, suggesting compensatory mechanisms [40].

Other less-studied clan CA members in *Leishmania* include Atg4.1 and Atg4.2 (clan CA, family C54) and otubain-like enzymes (clan CA, family C65). Atg4.2 knockout was associated with impaired metacyclogenesis, altered autophagosome accumulation (especially under nutritional stress), increased amastigote length, and impaired amastigote proliferation; in contrast, Atg4.1 knockout only showed minor effects on promastigote infectivity [41]. A novel otubain-like CP is a deubiquitinating enzyme recently characterized from *L. infantum* that may also have proinflammatory activity by stimulating lipid droplet biogenesis and inducing IL-6 and TNF-α secretion [42].

Early work with vinyl sulfone and dihydrazide compounds showed successful in vivo treatment of cutaneous leishmaniasis by CPC inhibition [43]. Since then, several other CPC inhibitors have shown in vivo efficacy to treat cutaneous (palladacycle DPPE 1.2 [44]; organotellurane RT-01 [45]) and visceral (organotellurane RF07 [46]) leishmaniasis. In vitro assays identified multiple compound classes active against CPB (e.g., semicarbazones, thiosemicarbazones, triazine nitriles [47], and benzophenones [48]), but these have yet to be tested in vivo.

Trypanosoma brucei

Human African trypanosomiasis (sleeping sickness), caused by various subspecies of *T. brucei*, is found in sub-Saharan Africa. The flagellated parasite invades the blood–brain barrier, causing fatal damage in the central nervous system, with approximately 7,000 deaths per year [20]. Similar to *Leishmania* CPs, the best characterized *T. brucei* CPs are cathepsin-like enzymes, cathepsin L (TbCatL, also known as brucipain and rhodesain in different *T. brucei* subspecies) and cathepsin B (TbCatB) [49].

TbCatL and TbCatB are both involved in virulence. TbCatL promotes parasite crossing of the blood–brain barrier via activation of host G-protein–coupled receptors such as PAR2 (protease-activated receptor 2) and subsequent induction of host calcium-signaling pathways [50, 51]. TbCatL also protects *T. brucei* from lysis by host serum [52, 53] and from opsonization via degradation of internalized variant surface-glycoprotein–bound antibodies [54]. As for TbCatL [55], TbCatB expression is greatest in the bloodstream stage [56]; it mediates the degradation of endocytosed host transferrin in the parasite endosomal/lysosomal compartment as part of *T. brucei* iron acquisition pathways [57, 58]. TbCatB may also be involved in cytokinesis [56]. A number of studies using RNA interference (RNAi) [58] or small-molecule inhibitors in vitro [59] and in animal infection models [60, 61] have validated these proteases as drug targets. K11777 in particular targets TbCatL: it showed synergy when combined with eflornithine and might be considered for development as a new therapy for African trypanosomiasis [62]. Other small molecules have been successfully tested against TbCatL, including dipeptide nitriles [63] and triazine nitriles [64], but their efficacy in vivo is yet to be determined. A recent review highlights a thorough list of candidate *T. brucei* and *T. cruzi* CP inhibitors [65].

Trypanosoma cruzi

Chagas disease is endemic in the Americas and is the main cause of heart failure in Latin America, leading to more than 12,000 deaths every year [66]. It is caused by the parasite *T. cruzi*, which encodes genes from four clans of CPs: CA, CD, CE, and CF. Clan CA includes the

most abundant protease of this parasite: cruzain (cruzipain), a papain-like cathepsin L-like member of family C1.

Cruzain is present in epimastigotes and bloodstream trypomastigotes and is identified as a major antigen in infected humans [67], and for that reason, it has been considered as a vaccine candidate [68]. It plays important roles in differentiation [69], metabolism [70], evasion of the immune response, and invasion of host cells [71]. In trypomastigotes (infective stage), cruzain is localized in the flagellar pocket, and in intracellular amastigotes, the enzyme is on the cell surface. Recombinant cruzain was expressed in bacteria and demonstrated protease activity after autocatalytic activation [72]. The crystal structure of cruzain complexed with the inhibitor Z-Phe-Ala-fluoromethyl ketone was resolved, confirming structural similarities with papain.

Genes coding for a 30-kDa cathepsin B-like CP, two other cathepsins, and other homologues of calpains (family C2) are also present [74]. Other members of clan CA include autophagin-like Atg4 protease (family of C54), responsible for processing Atg8 for the formation of the autophagosomes involved in the parasitic autophagy process essential for metacyclogenesis and virulence [75, 76]. Atg4 has recently been proposed as a potential new target for chemotherapy [76].

The idea of developing CP inhibitors as Chagas disease chemotherapy originated in observations of in vitro antiparasitic effects associated with cruzain inhibition. Because of multiple copies of the cruzain gene, gene knockout could not be achieved to confirm the essentiality of the enzyme, even though "chemical validation" with protease inhibitors suggested that it is essential. The first proof of concept in an animal model showed that mice could be rescued from a lethal *T. cruzi* infection with the vinyl sulfone inhibitor K11777 dosed for 20 days [3]. Surviving mice had negative hemoculture, indicating parasitological cure. The same inhibitor was later tested in a dog model of Chagas disease, and it prevented cardiomyopathy after 7 days of treatment [2].

A number of other cruzain inhibitors are under study. Computational screening of the ZINC database identified inhibitors with nanomolar potency against cruzain [77]. Some isatins also have the thiosemicarbazone functionality, a well-known cruzain inhibitor group [78]. However, isatin compounds without a thiosemicarbazone also demonstrated inhibitory activity against cruzain [79]. These compounds have a peptide sequence recognized by the catalytic site of cruzain as well as the epoxy electrophile reminiscent of the E-64 inhibitor. Odanacatib, a reversible inhibitor of human cathepsin K with an amino nitrile warhead, was in Phase III trials to treat osteoporosis, but its development was discontinued due to risk of stroke (see the associated chapter, "Cysteine proteases as digestive enzymes in parasitic helminths," for information on odanacatib efficacy against hookworm infection). The nitrile warhead forms a reversible (albeit with a slow off rate) thioimidate with the active site of human cathepsin K [80]. Inspired by this approach, reversible inhibitors have been developed against cruzain with 100× selectivity compared to human cathepsins L, B, S, and F. Further optimization of these compounds led to the discovery of Cz007 and Cz008, with IC_{50}s against cultured parasites in the nanomolar range and antiparasitic efficacy in a mouse model of Chagas disease [81]. More recently, the vinyl sulfone WRR-669 was demonstrated to be a noncovalent inhibitor of cruzain. These results, showing both in vitro and in vivo efficacy against *T. cruzi*, support cruzain as a valid and druggable target for Chagas disease. A review published in 2015 highlights inhibitors tested against cruzain [83].

Cryptosporidium parvum

Cryptosporidiosis, caused by *Cryptosporidium* sp., is a concern worldwide. In a large study in sub-Saharan Africa and southern Asia, this protozoan was the second most common cause of moderate-to-severe diarrhea in infants and the third most common cause in toddlers,

accounting for an estimated 2.9 and 4.7 million cases annually in children <2 years old in these regions, respectively [84]. Acquired immune deficiency syndrome (AIDS) patients, people under immunosuppressive treatments, patients with inheritable immunodeficiency, diabetic people, infants, and old or malnourished people are the most susceptible to severe cryptosporidiosis [85]. Outbreaks have been described among caregivers and students of veterinary hospitals after contact with calves infected with *C. parvum*, the zoonotic species [86]. A major outbreak was reported in Milwaukee in 1993, in which 403,000 people were infected and the cost of outbreak-associated illness was US$96.2 million [87, 88].

Genes encoding 20 clan CA cathepsin L-like proteases have been reported in *Cryptosporidium parvum*. Five genes coding for cryptopains—cathepsin L-like proteases, a representative of clan CA—were identified in the *C. parvum* genome [89], but only cryptopain-1 has been analyzed biochemically [90]. Cryptopain-1 is actively transcribed and expressed in sporozoites, the infectious stage of the parasite [90]. At physiologically achievable concentrations, K11777 arrested the growth of *C. parvum* in human intestinal cell lines [91]. In C57BL/6 interferon-γ receptor knockout mice, which are highly susceptible to *C. parvum* infection (mimicking cryptosporidiosis in AIDS patients), K11777 administered either orally or intraperitoneally rescued mice from a lethal *C. parvum* infection [91].This potent anticryptosporidial activity is hypothesized to be the result of K11777 inhibiting the active site of cryptopain-1 [91].

Recently, otubain protease (OTU), a CP that participates in the ubiquitin pathway, has also been identified [92]. The biochemical properties of the otubain-like CP of *C. parvum* (CpOTU) were characterized, and the enzyme may have an essential function during the oocyst stage of the parasite, when its expression reached maximum levels [93]. The protein contains an unusual C-terminal extension (217 amino acids) compared to other OTUs previously identified in human, mouse, and *Drosophila*, and deletion of the extension resulted in complete loss of enzyme activity.

Plasmodium sp.

Malaria is by far the deadliest parasitic disease of humans, with 446,000 or 631,000 [94, **95**] deaths estimated in 2015 using different modeling approaches. *Plasmodium falciparum* and *P. vivax* are the most common species infecting humans, with *P. falciparum* responsible for nearly all deaths. The parasites express multiple CPs, some of which are the subjects of recent reviews [96–98]. For *P. falciparum*, the genome sequence predicts 33 CP-like proteins, although a number of these are probably not active enzymes. The best characterized are 4 falcipains and 3 dipeptidyl peptidases, all clan CA proteases [96].

The functions of plasmodial CPs have been characterized using selective CP inhibitors and in some cases by gene knockout. Erythrocytic malaria parasites import erythrocyte cytosol and degrade hemoglobin in an acidic food vacuole as a source of amino acids [**99**] in a cooperative process involving enzymes of multiple catalytic classes, including CPs, aspartic proteases, and metalloproteases. Incubating parasites with broadly active CP inhibitors causes the food vacuole to swell and fill with undegraded hemoglobin, suggesting CP essentiality in hemoglobin processing. CPs also contribute indirectly to hemoglobin hydrolysis via the processing of plasmepsins to active enzymes [100]. The CPs with clear roles in hemoglobin hydrolysis are falcipain-2, falcipain-3, and dipeptidyl aminopeptidase 1. Knockout of falcipain-2 caused the food vacuoles of *P. falciparum* trophozoites to fill with undegraded hemoglobin, but this abnormality resolved later in the life cycle, presumably due to expression of falcipain-3 [101]. In contrast, knockout of falcipain-3 was not tolerated, suggesting that this protease is essential [102]. Some studies have also suggested roles for CPs in erythrocyte rupture at the completion of the erythrocytic cycle or in merozoite invasion of erythrocytes [103]. CP inhibitors blocked the

rupture of erythrocytes by mature schizonts. Mediators of this process are predicted to be members of the SERA family, including the pseudo-CP SERA5 [**104**], the functional CP SERA6 [105], and dipeptidyl aminopeptidase 3 [103, 106], although recent reports refute activity of dipeptidyl aminopeptidase 3 in erythrocyte rupture [98, 107]. Recent advances have demonstrated a proteolytic cascade responsible for egress of merozoites from host erythrocytes; this cascade includes cleavage of the actin-binding domain of the erythrocyte cytoskeletal protein β-spectrin by SERA6 to mediate erythrocyte rupture, the final step required for merozoite egress [108]. Some studies have also suggested that CPs participate in erythrocyte invasion by asexual parasites, notably falcipain-1 [**109**] and dipeptidyl aminopeptidase 3 [98]. However, studies with protease inhibitors have generally not supported this conclusion; knockout of falcipain-1 did not block *P. falciparum* development [**110, 111**], and antibodies against the endogenous *P. falciparum* CP inhibitor falstatin blocked invasion, suggesting that inhibiting falcipain-like CP activity facilitates invasion [112]. Considering another family of clan CA CP, a nucleolar calpain-like *P. falciparum* CP appears to be required for the development of erythrocytic parasites [113, 114]. Also, a *P. falciparum* otubain-like CP was recently shown to localize to the apicoplast organelle and to be required for normal apicoplast and parasite development via inhibition of the predicted role of *P. falciparum* Atg8 in protein import to the apicoplast [115].

CPs appear to play additional roles in nonerythrocytic plasmodial stages. Considering liver stages, an unidentified plasmodial CP cleaves the circumsporozoite protein, which coats the sporozoites injected by mosquitoes, to enable invasion of hepatocytes [116]. In the murine parasite *P. berghei*, the orthologue of falcipain-1 appears to be critical for invasion of erythrocytes by hepatocyte-derived merozoites [117]. Considering mosquito stages, CP inhibitors and the knockout of falcipain-1 decreased oocyst production in mosquitoes [110, 118]. Also, dipeptidyl aminopeptidase 2, which is expressed in gametocytes, may contribute to gamete egress [**119**].

Our understanding of the roles of CPs in the plasmodial life cycle suggests numerous potential drug targets. Falcipain-2 and falcipain-3 have low pH optima, consistent with activity in the acidic food vacuole, and both enzymes were localized to the food vacuole [120]. Falcipain-2 is more active against peptidyl substrates, uniquely able to activate and undergo autohydrolysis at neutral pH, and more stable at neutral pH. Considering specificity for peptide substrates and inhibitors, important differences were seen between falcipain-2, falcipain-3, and homologs from the rodent parasites *P. berghei* and *P. vinckei*; differences in specificity between falcipain-2 and falcipain-3 were less pronounced [121]. Both enzymes are synthesized as membrane-bound proforms that are processed, probably by autohydrolysis, to soluble mature forms. A related enzyme, falcipain-2′, is nearly identical in sequence and biochemical features to falcipain-2, but its role is uncertain, as in contrast to the case with falcipain-2 and falcipain-3, knockout of falcipain-2′ had no clear phenotype [122]. The falcipains have some unique features for papain-family proteases, including unusually long N-terminal domains and insertions in the catalytic domain. Identified functions of these domains include trafficking of falcipain-2 to the food vacuole by upstream portions of the prodomain; enzyme inhibition by downstream portions of the prodomain; mediation of enzyme folding by short peptides immediately upstream of the catalytic domain [**123**]; and mediation of binding to the native substrate, hemoglobin, by a small insertion near the C-terminus of the catalytic domain.

Multiple studies have demonstrated that CP inhibitors have potent antimalarial effects [124]. With these inhibitors, a block in *P. falciparum* development is accompanied by a specific block in hemoglobin hydrolysis, marked by the appearance of swollen, hemoglobin-filled food vacuoles, and antiparasitic effects correlated with the degree of inhibition of falcipain-2 and falcipain-3. Drug discovery directed against falcipains is facilitated by the available structures of falcipain-2 and falcipain-3 complexed with small-molecule and protein inhibitors [**125**].

Peptidyl falcipain inhibitors with nanomolar antimalarial activity have included fluoromethyl ketones, vinyl sulfones, and aldehydes; in some cases, in vivo activity against murine malaria has also been demonstrated [126]. Promising nonpeptidyl falcipain inhibitors have included a series of nitriles that was extensively studied with many promising features, including excellent in vitro and in vivo potency [127]; this project was halted because of tissue binding that might predict idiosyncratic toxicity, but the evaluation of nitrile inhibitors is ongoing. Another interesting approach is the optimization of natural products, including analogues of gallinamide A, a compound from cyanobacteria with nanomolar antimalarial activity [128], and sugarcane cystatin [129].

Concerning the potential for resistance, parasites were selected for resistance to a vinyl sulfone falcipain inhibitor, but the selection was slow and the mechanism of resistance complex, without mutations in target enzymes, suggesting that resistance to falcipain inhibitors may develop slowly, especially with combination therapy [130]. Considering combinations, the activity of artemisinins, the mainstay of modern treatment for falciparum malaria, requires falcipain activity [**131**]; thus, falcipain inhibitors should probably not be combined with artemisinins. In contrast, inhibitors of CPs and aspartic proteases showed synergistic antimalarial effects, consistent with a complementary role for these two classes of enzymes and suggesting the potential for synergistic combination antimalarial therapy [132].

Toxoplasma gondii

Toxoplasma gondii is a foodborne pathogen with seroprevalence ranging from 10%–30% in North America and northern Europe to more than 80% in areas of Latin America and Africa [133]. Most infected individuals remain asymptomatic despite lifelong infection. In contrast, congenital transmission or infection of immunocompromised patients with AIDS or organ transplants can lead to fatal, disseminated disease [133]. *T. gondii* CPs have been shown to be important for invasion, digestion of host proteins for nutrition [134, 137], and autophagy for cyst survival [138]. The *Toxoplasma* genome project revealed that the redundancy of CP genes is lower in *T. gondii* than in most other studied parasites. For example, *E. histolytica* has more than 50 genes encoding CPs with similar structure and specificity [139]. In contrast, *T. gondii* has genes encoding only one cathepsin B (TgCPB), one cathepsin L (TgCPL), and three cathepsin Cs (TgCPC1, 2, and 3), potentially making them more amenable drug targets. Active recombinant proteases have been expressed for all the *T. gondii* CPs, simplifying structure-based drug design.

TgCPB and TgCPL have been linked to host cell invasion. Targeting TgCPB with a peptidyl cathepsin B inhibitor or antisense RNA inhibited host cell invasion and in vitro growth and blocked infection in a chick embryo model of toxoplasmosis [142]. TgCPL acts as a maturase for TgCPB [**143**] and key adhesins, the microneme proteins MIC2-associated protein (M2AP) and MIC3. TgCPL knockouts were attenuated in virulence in acute infection in mice [134]. Both TgCPB and TgCPL have been localized in the vacuolar compartment, a lysosome-like organelle, where TgCPL has been shown to digest host cytosolic proteins. Most recently, TgCPL has been shown to be important for cyst survival in latent infection [138]. In TgCPL knockout strains or cysts incubated with the vinyl sulfone inhibitor LHVS, autophagy of autophagosomes in the vacuolar compartment was inhibited, resulting in abnormal cyst morphology and decreased survival.

The most developed peptidyl inhibitors target TgCPB and/or TgCPL. The crystal structure of TgCPL with morpholinurea-leucyl-homophenyl-vinyl sulfone has been determined, and the inhibitor can block host cell invasion [136] and cyst viability in vitro [138]. K11777 inhibits purified recombinant TgCPB and TgCPL in the nanomolar range and blocks host cell

invasion, parasite replication, and viability in a chick embryo egg model [144]. Unfortunately, neither vinyl sulfone inhibitor is likely to cross the blood–brain barrier to prevent latent infection, so further optimization will be required.

The *T. gondii* cathepsin Cs are exopeptidases that are also potential drug targets. TgCPC1 was the most highly expressed cathepsin mRNA in tachyzoites; TgCPC3 was only identified in oocysts [137]. Both TgCPC1 and TgCPC2 localize to the dense granules and are secreted into the parasitophorous vacuole, where they degrade peptides. Both TgCPC1 and TgCPC2 were inhibited by Gly-Phe-dimethylketone, reducing parasite intracellular growth and proliferation. The same phenotype was not seen with a TgCPC1 knockout, as TgCPC2 expression was up-regulated, suggesting the importance of inhibiting both enzymes [137].

Autophagy is a key process in all eukaryotic cells to remove and recycle misfolded proteins and damaged organelles. Autophagy is likely to be important in *T. gondii* tachyzoites to survive extracellular stress and for bradyzoites during latent infection [138]. Although only a limited number of autophagy proteins (Atg) are encoded in the *T. gondii* genome, and there are no classic lysosomes in this organism, autophagosome-like bodies are formed [138, 145]. TgCPL appears to play an important role in the degradation of autophagosomes, as knockout or inhibition of TgCPL results in undigested proteins and organelles in the vacuolar compartment, limiting chronic infection in mice.

The clan CA cysteine proteinases of *T. gondii* have been identified as potential vaccine candidates. DNA vaccines containing TgCPB or TgCPL gene sequences individually or together produced both humoral and cellular immune responses in BALB/c mice. Following immunization, survival was prolonged following intraperitoneal challenge with tachyzoites, with the most significant effect from the combined TgCPB/TgCPL vaccine [146]. Similar results were seen with a TgCPC1 DNA vaccine [147].

Conclusion

From the 7 protozoan parasites causing human disease that are of interest in this review, only 2 genera (*Trypanosoma* and *Plasmodium*) have at least one well-characterized clan CA CP: cruzain in *T. cruzi*, TbCatL in *T. brucei*, and falcipain-2 and falcipain-3 in the malaria parasite. These enzymes were validated as drug targets using a variety of inhibitor chemistries.

One common theme is the observation that distinct pathogens employ related CPs to perform similar functions. For example, the process of invading a tissue in the case of extracellular parasites [10, 19, 51, 148] and invading a host cell in the case of intracellular parasites [71, 135, 136, 140] is highly dependent on clan CA proteases. It is also interesting to note that parasites have evolved different mechanisms to utilize CPs to modulate the immune system of the host. EhCP can directly degrade IgA, IgG, and IL-18 [11–15]. By contrast, CPB in *Leishmania* sp. modulates host responses by down-regulating protective Th1 immune responses, and in particular IFN-γ production, via degradation of the transcription factor NF-κB and the subsequent inhibition of IL-12 production by infected macrophages [26, 35, 149].

Apart from the more well-studied *Trypanosoma* and *Plasmodium* sp., it is important to continue investigations regarding the therapeutic potential of other protozoan CPs. In support of this, essentiality has been suggested or demonstrated for the CPs in many of the species discussed here. Furthermore, the potential for the emergence of other protease targets is great, considering that less than 10% of putative CPs found in the respective genomes have been so far characterized.

Key learning points

- Gaps in our knowledge: there are many genome copies of CPs per protozoan organism. Less than 10 enzymes have been well characterized per parasite and not more than 2 enzymes per pathogen have had their structures resolved by X-ray crystallography.
- Importance: the major functions of CPs shared by these protozoan parasites are host invasion and tissue penetration, virulence and evasion/modulation of the host immune system.
- Potential targets for chemotherapy: the most well studied CPs of protozoan parasites are the cathepsin L-like enzymes from *T*. *brucei* and *T*. *cruzi*, and falcipain-2 and falcipain-3 from *Plasmodium* sp. They are validated targets in vitro and in vivo for the development of novel therapies.

Top four papers

1. McKerrow JH. Development of cysteine protease inhibitors as chemotherapy for parasitic diseases: insights on safety, target validation, and mechanism of action. Int J Parasitol. 1999;29(6):833–7. PubMed PMID: 10480720. [**162**]
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