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Cysteine Protease Inhibitors and Parasitic Diseases

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ABBREVIATIONS

BBB: Blood brain barrier, **CP:** Cysteine protease, **CPI:** cysteine protease inhibitor, **E-64:** Epoxid compound: Clonazepam©, **E/S:** Excretory-secretory, **FP2:** Falcipain-2**HDAC:** Histone deacetylases, **IFN:** Interferon, **LC:** Lactacystin, **NO:** Nitrous oxide, **PCD:** Programmed cell death, **PMN:** Polymorphonuclear neutrophil granulocytes, **PMSF:** Phenyl-methyl-sulfonyl fluoride, **PVS:** Polyvenyl sulfone, **SBTI:** Soybean trypsin inhibitor, **SLA:** Soluble *Leishmania* antigen, **TGF:** Transforming growth factor, **TLCK:** Tosyl lysine chloromethyl ketone, **TNF:** Tumor necrosis factor, **TPCK:** Tosylamide phenylethyl chloromethyl ketone, **VS:** Vinyl sulfone.

INTRODUCTION

Proteases (peptidases) are enzymes for proteins catabolism through the cleavage of peptide bonds. Biochemically, they are divided into 2 broad types, the 1st of which does not form a transient covalent bond with the peptidase, but breaks the peptide bond either with the aid of a metal cation (metallo-peptidase) or without it (aspartyl-peptidases). The 2nd type forms a transient covalent bond of serine (serine peptidases) or threonine (threonine peptidases), or the sulfur of an essential cysteine (cysteine peptidases, CPs)[1]. The database internet site, MEROPS, classifies all known peptidases, and includes information on peptidase inhibitors, specificity and their extensive summaries. In this database, there are 9 classes of CPs referred to as clans, and one unclassified clan. The majority of parasitic CPs are included in 2 clans**:** CA (lysosomal cathepsins) and CD (caspase). Four other clans of registered proteases are related to parasites such as *T. gondii* (Clan CE; adenain), *T. vaginalis* (Clan CO; dipeptidyl peptidase VI), and both *B. malayi* and *T. spiralis* (Clan CH; hedgehog protein)[2].

In the early 80s, proteinase inhibitors were separated into 2 general categories based upon their spectrum of activity**:** the non-specific and specific proteinase inhibitors[3,4]. According to the researchers' overview, non-specific proteinase inhibitors were capable of inhibiting members of all classes of proteinases, and consist solely of the α-macroglobulins. On the other hand, class-specific proteinase inhibitors are each capable of inhibiting only one class of proteinases. The higher specificity of proteinase inhibitors in this class is due to specific binding sites located within the active site of the inhibitor. Later in 2007, the inhibitors were generally reclassified into 2 large groups based on their structure**:** low molecular weight peptidomimetic inhibitors and protein protease inhibitors composed of one or more peptide chains[5]. In the present review, most of the researched studies used the old classification of specific and non-specific CPIs.

In the literature review, the most commonly used CPIs are vinyl sulfones (VSs) and histone deacetylases (HDACs). Sequence analyses and substrate profiling identified cruzain, rhodesain and falcipain-3 as cathepsin L-like, and several studies described classes of small molecule inhibitors that target multiple cathepsin L-like CPs, some with overlapping anti-parasitic activity[6,7]. Among these small molecules, VSs proved to be effective inhibitors of a number of papain family-like CPs[8,9]. The group of HDAC forms a conserved enzyme family that controls gene expression *via* the removal of acetyl residues from histones and other proteins and are under increasing investigation as therapeutic targets, notably in cancer and parasitic diseases[10]. This group is classified into 4 classes (I) HDAC1-3 and HDAC8, (II) HDAC4-7, HDAC9 and HDAC10, (III) 7 sirtuin members (SIRT1-7)

and (IV) HDAC11 which has some similarity with class-I HDACs[11]. It was found that class-I HDACs are primarily localized in the nucleus, while class-III HDACs have more tissue specificity and can be located in the nucleus and cytoplasm. It was also observed that class-III HADCs have a role in many cellular functions, including gene repression, apoptosis, DNA repair and promotion of longevity[12]. Moreover, HDACs have been identified in all the major human parasitic pathogens[13]. Examples of HDACs are trichostatin A (TSA), valproic acid (VPA) and sodium-butyrate (BA). The 1st has the ability to induce cell cycle arrest, cell differentiation and apoptosis[14]. The 2nd is one of the simplest drugs currently available in therapeutic arsenal for the treatment of migraine prophylaxis, bipolar disorder and epilepsy[15]. The 3rd HADC inhibitor (BA) has long been known as a non-specific gene activator[16].

The present review aims to through light on different parasitic CPs that could be inhibited by general and/or specific CPIs. The present review will help researchers to select between different CPIs in their research projects with parasites.

Keywords: Parasites, MEROPS, Cysteine Protease, Chagasin, Papain, Cathepsin, Calpain, Cysteine Protease Inhibitors Calpeptin, E-64, K11777, Leupeptin, TLCK, TPCK, VS compounds.

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Cysteine Protease Inhibitors for Parasites

Entamoeba histolytica

In the year 2000, it was reported that *E. histolytica* CPs are encoded by at least seven genes, several of which are not found in *E. dispar*. A number of animal models proved useful to confirm the critical role of CPs in invasion. The investigators claimed at that time that detailed structural analysis of these CPs should provide further insights into their biochemical function and can facilitate the design of specific inhibitors which could be used as potential future chemotherapeutic agents[17].

A crucial role for amoebic CPs was shown by prevention of villin proteolysis and associated microvillar alterations. This was achieved by treatment of trophozoites before co-culture with synthetic inhibitors that completely blocked amoebic CP activity on zymograms. Moreover, trophozoites of amoebic strains (pSA8 and SAW760) with strongly reduced CP activity showed reduced proteolysis of villin in co-culture with enteric cells[18].

Entamoeba histolytica contains at least 50 CPs; however, only three (EhCP1, EhCP2 and EhCP5) are responsible for approximately 90% of the CP activity in this parasite. Cysteine proteinases play a central role in tissue invasion, disruption of colonic epithelial barrier and disruption of host defenses by digesting components of the extracellular matrix and immunoglobulins, through cleavage of secretory immunoglobulin A (sIgA), IgG, and activation of complement and cytokines[19].

There are more than 40 genes encoding *E. histolytica* CPs[20]. In 2010, however, it was shown that the 50 EhCPs are encoded by 50 genes, of which EhCP4 (EhCP-4a) is the most up-regulated during invasion and colonization in a mouse cecal model of amoebiasis[21]. In their article, the investigators found that specific VS inhibitor (WRR605) synthesis based on the substrate specificity of EhCP4, inhibited the recombinant enzyme *in vitro* and significantly reduced parasite burden and inflammation in the mouse cecal model. The unique expression pattern, localization and biochemical properties of EhCP4 could be exploited as a potential target for drug design[21].

As *E. histolytica* CP1 (EhCP1) is highly expressed and released, a new VS inhibitor (WRR483) was synthesized based on its specificity to target EhCP1. The effects of K11777 as VS inhibitor and WRR483 on invasion of human colonic xenografts were tested. The resultant dramatic inhibition of invasion by both inhibitors in human colonic model of amoebiasis strongly suggests a significant role of secreted amoebic proteinases, such as EhCP1, in the pathogenesis of amoebiasis[20].

Effects of CPIs on amoeba strains not expressing pathogenic factors (amoebapore A and cysteine protease A5) indicated that cell death and cytoskeleton disorganization depend upon parasite adhesion and amoebic CP activities. Data reported from France on amoebae interactions with human hepatic endothelial cells, established a relation between cytotoxic effects of *E. histolytica* and altered human target cell adhesion and suggested that interference with adhesion signaling triggers endothelial cell retraction and death[22]. On the other hand, caspase is a CP that plays a central role in the execution-phase of cell apoptosis. Administration of the pan caspase inhibitor (ZVAD) decreased the rate and severity of amoebic infection in CBA mice by all measures (cecal culture positivity, parasite enzyme-linked immunosorbent assay and histological scores) [23]. In another study conducted in Korea, the researchers investigated whether calpains (calcium-dependent, non-lysosomal CPs) are involved in the *E. histolytica*-induced cell death of HT-29 colonic epithelial cells. When HT-29 cells were co-incubated with *E. histolytica*, the propidium

iodide-stained dead cells were markedly increased as compared to that in HT-29 cells incubated with medium alone. This pro-death effect induced by amoeba was effectively blocked by pretreatment of HT-29 cells with the calpain inhibitor, calpeptin[24]. In addition, the involvement of the protein tyrosine phosphatases (PTPs), SHP-1 and SHP-2 in the dephosphorylation associated with *E. histolytica*-induced host cell death was investigated[25]. The investigators found that their incubation induced marked decrease in the protein tyrosine phosphorylation levels and SHP-1 or SHP-2 in Jurkat T cells. When cells were pre-treated with a calpain inhibitor (calpeptin), hindered the amoeba-induced dephosporylation and cleavage of SHP-1 or SHP-2. Moreover, PTPs inhibition with phenylarsine oxide (PAO) attenuated *Entamoeba*-induced dephosphorylation and DNA fragmentation in Jurkat T cells[25].

Cysteine proteases inhibitors of the chagasin-like inhibitor family (MEROPS family I42) were recently identified in bacteria and protozoan parasites. A CPI, EhCPI1, with significant homology to chagasin was identified; and the recombinant EhCPI1 was found to inhibit the protease activity of papain and that of a trophozoite lysate[26]. The investigators claimed that it may be a candidate for the rational development of anti-amoebiasis drugs. Moreover, it was found that *E. histolytica* contains two CPI-encoding genes of the chagasin-like inhibitor family (EhCPI1 and EhCPI2). They are also known as amoebiasin 1 and 2[19]. In 2012, a study was conducted in Germany investigating the tasks performed by both CPIs in the regulation of endogenous protease activity in *E. histolytica* trophozoites. It was found that EhCPI1 was localized to the cytosol, whereas EhCPI2 was targeted to phagosomes. The investigators proposed that EhCP-A1 accidentally released into the cytosol is the main target of EhCPI1, whereas EhCPI2, beside its role in house-keeping processes, may control the proteolytic processing of other hydrolases or fulfil other tasks different from protease inhibition[27].

Giardia lamblia

It was found that allicin, one of the active principles of garlic homogenates exhibits antiparasitic activity due to its chemical reaction with thiol groups of various enzymes, affecting essential metabolism of CP activity involved in *G. lamblia* virulence[28]. On the other hand, it was shown that there are two proteins (CWP1 and CWP2) involved in formation of *G. lamblia* cyst wall; and that specific inhibitors prevent release of these cyst wall materials thus abolishing cyst wall formation. The encystation-specific CP responsible for the proteolytic processing of CWP2 is homologue to lysosomal cathepsin C. These features provided new insights regarding cyst wall formation in *Giardia* [29]. Another study showed that *G. lamblia* induces enterocyte apoptosis in duodenal epithelial monolayers increasing epithelial permeability which could be prevented by pretreatment with the caspase-3 inhibitor. These findings indicated that strain-dependent induction of enterocyte apoptosis may contribute to the pathogenesis of giardiasis[30].

In 2006, a study conducted in Mexico showed that the proteolytic activity of *G. lamblia* trophozoites was enhanced on *in vitro* co-culture with IEC6 cells. This activity was strongly inhibited by an epoxid compound (E-64) and L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) as CPIs, and a concomitant inhibition of parasite adhesion to IEC6 cells was observed. These data suggested that trophozoites secrete CPs that play a role in *G. lamblia* adhesion to epithelial cells[31]. In another study conducted in Egypt, the investigators showed that *G. lamblia* cysts incubated with E64 *in vitro* completely failed to excyst compared to 90% that completely excysted when incubated without E-64. *In vivo* evaluation of the therapeutic response proved that there was decrease in the cysts out-put in the stools of infected treated mice in comparison to untreated mice[32].

***Cryptosporidium* species**

It was shown that an azocasein proteinase found on the surface of *C. parvum* sporozoites was inhibited by ethylene-diamino-tetra-acetic acid (EDTA), iodoacetic acid, E-64 and phosphoramidon[33]. Another study showed that the combination of phenyl-methyl-sulfonyl fluoride (PMSF) and E-64 inhibited >95% of the azocasein hydrolysis, but had no inhibitory effect on oocyst excystation[34]. The same authors investigated the anti-cryptosporidial potential of several protease inhibitors including antipain, aprotinin, leupeptin, soybean trypsin inhibitor (SBTI) and PMSF in a cell culture system. Parasite number was reduced to 40-50% in leupeptin, SBTI and PMSF, and to 10-15% in antipain and aprotinin. These findings suggested that a protease component of *C. parvum* may be essential for host cell infection[35]. In addition, treatment of *C. parvum* oocysts with hydrogen peroxide inhibited protease activity up to 50% compared with untreated controls, while their treatment with chemicals that affect sulf-hydryls inhibited protease activity by > 90%. Oocysts treatment with these chemicals, along with the protease inhibitors (PMSF, EDTA and cystatin) inhibited protease activity as well as *in vitro* infection and excystation[36].

It was found that apoptosis of epithelial cells could be involved in the pathogenesis of cryptosporidiosis *in vivo* due to caspase inhibition[37]. In 2008, a study showed

that cryptosporidiosis resulted in low-level activation of multiple members of the caspase family, and caspase activation kinetics was correlated with apoptosis. Furthermore, cryptosporidiosis led to up-regulation of genes encoding inhibitors of apoptosis proteins (IAPs) and survivin[38].

The impact of cryptosporidiosis on host cell gene expression was investigated. In early infections (6 and 12 h), genes with anti-apoptotic roles were up-regulated and genes with apoptotic roles were down-regulated. Later on in infection (24, 48 and 72 h), pro-apoptotic genes were induced and anti-apoptotic genes were down-regulated, suggesting a biphasic regulation of apoptosis[39].

Recently, it was shown that *C. parvum* induces formation of an actin-dense plaque which is essential for the successful invasion of the host epithelial cells, which induced calpain activation. Inhibition of calpain activity by over expression of the endogenous inhibitor (calpastatin) diminished the formation of the actin-dense plaque and decreased the initial invasion of parasites[40].

Trichomonas vaginalis

It was reported that *T. vaginalis* proteinases are closely related to its pathogenicity and cytotoxicity[41]. The role of CPs in adherence of *T. vaginalis* to human vaginal epithelial cells was evaluated. Pretreatment with CPIs**:** N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), Leupeptin and TPCK greatly diminished its ability to recognize and bind to epithelial cells with inability to kill host cells[42]. On the other hand, pretreatment of complement-resistant parasites with CPIs resulted in lysis by complement, indicating that resistance was likely due to proteinase degradation of C3 on the trichomonal surface[43].

The role of cysteine and serine proteinase inhibitors (E-64, antipain, iodoacetic acid, iodoacetamide, TLCK) in reducing *T. vaginalis* ability to cleave Igs was evaluated. The proteinase activity and cytotoxicity of *T. vaginalis* to HeLa cells were decreased when live trophozoites were treated with metallo-proteinase inhibitor as well as cysteine and serine proteinase inhibitors. It was further explained that the proteinase cleaving ability of host Igs may be a contributing factor in the immune evasion mechanism for parasite survival in the host[44]. On the other hand, E-64 can inhibit *T. vaginalis* 39-kDa proteinase (CP39), which degraded collagens I, III, IV and V, human fibronectin hemoglobin and IgA and IgG[45]. Moreover, *T. vaginalis* CPs degrade secretory leukocyte protease inhibitor (SLPI) and render it nonfunctional. SLPI appears to prevent transmission of HIV through inhibition of virus entry into monocytic cells *in vitro*. So, the degradation of SLPI in association with trichomonal infection may increase the risk of HIV acquisition[46].

It was also shown that *T. vaginalis* secretes 5 proteinases that possess mucinase activity and that may be inhibited with CPIs. Adherence to soluble mucin prevented *T. vaginalis* attachment to HeLa cells. In addition, proteinase activity, adherence and motility were required to traverse a mucin layer *in vitro*[47]. In 2010, the Mexican investigators showed that *T. vaginalis* has many CPs, some of which are involved in its pathogenesis. Nine CPs were identified in the 30 kDa region (TvCP1, TvCP2, TvCP3, TvCP4, TvCP4-like, TvCP12, TvCPT, TvLEGU-1, and another legumain-like CP). The major reactive spots to *T. vaginalis*-positive patient sera corresponded to 4 papain-like (TvCP2, TvCP4, TvCP4-like, TvCPT), and one legumain-like (TvLEGU-1) CPs[48].

Toxoplasma gondii

Lactacystin (LC), a specific inhibitor of proteasomes in eukaryotic cells, inhibits parasite growth and daughter cell budding, as well as DNA synthesis. Two other proteasome inhibitors (MG-132 and proteasome inhibitor 1) were also found to block parasite growth and intracellular development. Adding LC to established dividing parasites rapidly blocked parasite growth and daughter cell budding, while pre-treating host cells with LC lead to parasite morphological changes. These changes were specific to LC and were not seen in parasites treated with other protease inhibitors. These results highlight the possible role of proteasome activity in *Toxoplasma* intracellular development and the regulation of parasite replication[49].

Studies showed that host cell apoptosis is inhibited by toxoplasmosis[50]. The investigators induced apoptosis in human-derived HL-60 and U937 cells by treatment with actinomycin D or TNF-α in combination with cycloheximide, respectively. Cleavage of caspases 3 and 9 was considerably diminished by *T. gondii*. It was suggested that *T. gondii* down regulates host cell apoptosis, through inhibition of cytochrome c release and subsequent caspase activation. However, it was shown that *T. gondii* inhibits apoptosis in infected cells by both caspase inactivation and transcription factor NF-kappa B activation[51].

Cysteine proteases play key roles in apicomplexan biogenesis, invasion and intracellular survival, and *T. gondii* cathepsin B is required for parasite invasion into cells. Processing of the pro-rhoptry protein 2 to mature rhoptry proteins was delayed by incubation of extracellular parasites with a cathepsin B inhibitor[52]. A study designed in USA, showed that three cathepsin inhibitors (III, TPCK and subtilisin inhibitor III) caused extensive swelling of the secretory pathway of the parasite leading to breakdown of the parasite surface membrane, disrupted rhoptry formation and accumulation of

abnormal materials in the parasitophorous vacuole as a result of proteolytic modification or degradation[53].

In 2007, it was reported that in *T. gondii* the papain family cathepsins**,** encoded by 5 genes including 3 cathepsin C (TgCPC1, 2 and 3), one cathepsin B (TgCPB), one cathepsin L (TgCPL) and TgCPC1, were the most highly expressed in tachyzoites. The specific cathepsin C inhibitor (Gly-Phe-dimethylketone) reduced parasite intracellular growth and proliferation, limiting the *in vivo* infection in the chick embryo model of toxoplasmosis. The targeted disruption of TgCPC1 does not affect tachyzoites invasion and growth, as TgCPC2 is then up-regulated and may substitute for TgCPC1. *T. gondii* cathepsin Cs are required for peptide degradation in the parasitophorous vacuole. Thus, cathepsins Cs are critical to *T. gondii* growth and differentiation, and their unique specificities could be exploited to develop novel chemotherapeutic agents[54]. On the other hand, it was shown that there are two genes encoding endogenous CPIs (toxostatins), which are active against both TgCPB and TgCPL. Over expression of toxostatin-1 decreased CP activity with no detectable effects on invasion or intracellular multiplication. These findings provided important insights into the proteolytic cascades of *T. gondii* and their endogenous control[55].

A series of selective CPIs were studied for their effects on *T. gondii* cell invasion. Two of these compounds (polyvinyl sulfones derivatives; PVS) impaired *T. gondii* invasion and gliding motility[56]. On the other hand, it was shown that *T. gondii* cysteine protease cathepsin L (TgCPL) is the primary target of one of these PVS derivatives[57].

Plasmodium species

Plasmodium trophozoite stages feed on host erythrocytes and release heme from globin within an acid food vacuole[58]. Cysteine protease inhibitors can block parasite hemoglobin hydrolysis and development, indicating that CPs are required for these processes[59].

Three papain-family CP sequences have been identified in the *P. falciparum* genome, falcipain-1(FP1) is expressed by erythrocytic parasites and it is not essential for normal development during erythrocyte invasion[60]. Falcipain-2 (FP2) was identified as a principal trophozoite CP and potential drug target, while falcipain-3(FP3) is expressed by trophozoites and appears to be located within the food vacuole, the site of hemoglobin hydrolysis. Thus falcipain-3 is a second *P. falciparum* hemoglobinase that is particularly suited for the hydrolysis of native hemoglobin in the acidic food vacuole[61]. It was shown that disruption of FP2 gene led to a transient block in hemoglobin hydrolysis with increased sensitivity to protease inhibitors. On the other hand, disruption of the FP3 gene is not possible strongly suggesting that this protease is essential for erythrocytic parasites. Disruption of the falcipain-1 gene did not alter erythrocytes development, but led to decreased production of oocysts in mosquitoes[62].

In 1996, the antimalarial effects of VS inhibitors were evaluated and were shown to strongly inhibit falcipain[63]. Similar results were obtained using 39 new VSs, as well as vinyl sulfonate ester and vinyl sulfonamide CPIs, suggesting that peptidyl VSs were promising antimalarial agents[64]. In the same year, activities of synthetic peptidyl aldehyde and alpha-ketoamide CPIs were evaluated against cultured *P. falciparum* and in a murine model. Results showed that these compounds inhibited FP2 and FP3 and blocked hemoglobin hydrolysis[65].

Falcipain-2-knockout trophozoites that markedly diminished CP activity were about 3 times more sensitive to CPIs (E-64 and leupeptin), and over 50-fold more sensitive to the aspartic protease inhibitor (pepstatin)[66]. Similar results were obtained using E-64d and EGTA-AM inhibitors. Both inhibitors prevented RBCs rupture, altered schizont morphology and supported parasitophorous vacuole breakdown[67]. On the other hand, circumsporozoite protein (CSP) is proteolytically cleaved by a papain family cysteine protease of parasite origin. Inhibitors of CSP processing inhibit cell invasion *in vitro*, and treatment of mice with E-64 completely inhibits sporozoite infectivity *in vivo*[68]. In 2007, evaluation of the role of E64d in inhibition of *P. falciparum* oocyst production showed significant inhibition of oocysts from 80 to 100%. In this study, only FP3 and not falcipain-2 was found to be expressed in stage V gametocytes. Interestingly, during gametocytogenesis FP3 was transported into the infected RBCs and by reaching stage V, it was localized in vesicles along the RBC surface, suggesting that that future drug design should include evaluation of gametogenesis and sporogonic development[69].

Falstatin, expressed in *Escherichia coli*, proved to be a potent reversible inhibitor of the *P. falciparum* CPs (FP2 and FP3), but it was a relatively weak inhibitor to falcipain-1. Falstatin is present in schizonts, merozoites and rings, but not in trophozoites, the stage at which the CPs activity is maximal. Falstatin is released upon the rupture of mature schizonts. Treatment of late schizionts with antibodies that blocked the inhibitory activity of falstatin decreased the subsequent invasion of erythrocytes by merozoites. These results suggested new strategies for the development of antimalarial agents that specifically disrupt erythrocyte invasion[70].

In 2011, novel dihydro-artemisinin derivatives were designed and synthesized as potential FP2 inhibitors.

The compounds showed excellent inhibition activity against *P. falciparum* FP2[71]. In another study in Italy, small peptides that mimic the protein-protein interactions between falcipain-2 and egg white cystatin, an endogenous inhibitor of CPs, were designed and synthesized and their effects on FP2 activity showed inhibition and produced morphological abnormalities in the *Plasmodium* food vacuole. This approach could be an interesting starting point for the development of a new class of anti-malarial drugs[72].

In a study conducted in 2012 Chinese scientists designed and synthesized a small molecular dual inhibitor based on the lead compound 1 of FP2 and dihydrofolate reductase as antimalarial agent. Six compounds showed improved dual inhibitory activities against FP-2. Molecular modeling provided the key structural information to maintain the dual inhibitory activity, and was helpful for future dual inhibitors design[73]. It was shown that N-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), a calpain inhibitor, showed an excellent inhibitory effect on the erythrocytic stages of *P. falciparum*[74]. In addition, selected gold compounds were found to cause pronounced inhibition of FP2 and effectively block *P. falciparum* growth *in vitro*[75].

In a study conducted in USA, Na *et al.*[76] identified and cloned genes encoding the *P. vivax* CPs, vivapain-2 and vivapain-3. These genes predicted papain-family CPs, and the vivapains were inhibited by fluoromethylketone and vinyl sulphone inhibitors that also inhibited falcipains and demonstrated potent antimalarial activities[76].

BDA-410 is another CPI that was evaluated *in vitro* and *in vivo* using *P. chabaudi* infection rodent model. BDA-410 inhibited parasite growth *in vitro* through irreversible damage to the intracellular parasite. *In vivo*, the BDA-410 significantly delayed the progression of malaria infection[77].

Finally, exoerythrocytic *P. berghei* parasites were found to express a potent CPI (PbICP), that has an important function in sporozoite invasion and is capable of blocking hepatocyte cell death. PbICP is secreted by sporozoites prior to and after hepatocyte invasion, localizes to the parasitophorous vacuole as well as to the parasite cytoplasm in the schizont stage and is released into the host cell cytoplasm at the end of the liver stage[78].

Leishmania species

Leishmania CPs are essential for parasite growth, differentiation, pathogenicity and virulence and are thus attractive targets for combating leishmaniasis. It was found that cathepsin L is one of the candidate endo/lysosomal enzymes in processing of soluble *Leishmania* antigen (SLA). Treatment of BALB/c mice with CLIK148 (Cathepsin L specific inhibitor) exacerbated the infection by enhancing the development of SLA-specific Th2-type response such as production of IL-4 and generation of Th2-dependent specific IgE/IgG1 antibodies[79]. On the other hand, transforming growth factor (TGF)-β is a potent regulatory cytokine that suppresses expression of inducible nitrous oxide (NO) synthase and interferon (IFN)-γ, and suppresses Th1 and Th2 cell development. Locally activated TGF-β enhances parasite survival through its effects on innate and adaptive immune responses. TGF-β activation by *Leishmania* was prevented by the specific cathepsin B inhibitor (CA074)[80].

A recombinant *L. mexicana* CP (CPB2.8) when inoculated into BALB/c mice, it up-regulated IL-4 and IL-4 production and induced strong specific IgE responses in treated mice. Inhibition of CPB2.8 activity by treatment with E-64 ablated the enzyme's ability to induce IgE[81]. It was shown also that deletion of the entire CPB gene array in *L. mexicana* was associated with decreased parasite virulence. These data indicated that *L. mexicana* CPs are critical in suppressing protective immune responses and that inhibition of CPB may prove to be a valuable immunomodulatory strategy for chronic forms of leishmaniasis[82]. The antiparasitic activity of VS compounds (isoxazoles and oxadiazole) as CPIs were shown to be potent reversible inhibitors to the recombinant *L. mexicana* CPB2.8[83]. Recently, CPB from *Leishmania spp.* represented an important virulence factor[84]. In *L. mexicana* infections, it was confirmed that CPs are virulence factors and CPIs have therapeutic potential effects[85].

In 2008, a study was conducted to investigate whether the blockade of caspase-8 activity would affect the expression of type-1 or type-2 cytokines. On early infections, both CD4 and CD8 T cells expressed IFN-γ upon activation. Treatment with the caspase-8 inhibitor reduced the proportion of CD8 T cells and IFN-γ expression in both CD4 and CD8 T cells. It was concluded that a non apoptotic role of caspase-8 activity may be required for T cell-mediated type-1 responses during *L. major* infection[86]. In the concept of apoptotic role in leishmaniasis, co-incubation of polymorphonuclear neutrophil granulocytes (PMN) with *L. major* promastigotes resulted in decrease in apoptotic neutrophils with reduction of caspase-3 activity in PMN. The inhibition of PMN apoptosis depended on the viable parasites[87]. Meanwhile, staurosporine, that induces apoptosis in all mammalian nucleated cells, also induces in *L. major* a death process with several cytoplasmic and nuclear features of apoptosis. It was proved that the induced apoptosis in *L. major* could be prevented[88].

Similarly, *in vitro* infection with *L. major* protects murine bone marrow-derived macrophages against programmed cell death (PCD) and delays PCD caused by treatment with staurosporine. The investigators concluded that the capacity of *L. major* to delay PCD induction in the infected macrophages may have implications for *Leishmania* pathogenesis by favoring the invasion of its host and the persistence of the parasite in the infected cells[89]. In another study conducted in USA, the researchers characterized two *L. donovani* metacaspases; LdMC1 and LdMC2. Consistently, LdMCs activity was found to be insensitive to caspase inhibitors and was efficiently inhibited by trypsin inhibitors, such as leupeptin, antipain and TLCK. In addition, LdMCs activity was induced in parasites treated with hydrogen peroxide, a known trigger of PCD in *Leishmania*. These findings suggest that LdMCs are effector molecules in *Leishmania* PCD[90]. The role of heat shock protein 90 inhibitor (geldanamycin; GA) during *L. donovani* promastigote-to-amastigote transformation was investigated. Results showed that GA could cause apoptosis in *L. donovani* but could not cause stage differentiation in high temperature and that acidic conditions were likely to be crucial for the transformation and survival of the parasite within its human host[91].

In addition, a study conducted in UK showed that CPI has a role other than modulation of the activity of the parasite's own CPs and their normal trafficking to the multi-vesicular tubule via the flagellar pocket. These results suggested that CPI has a role in protection of the parasite against the hydrolytic environment of the sandfly gut and/or the parasitophorous vacuole of host macrophages[92]. On the other hand, CPIs (aziridine-2,3-dicarboxylates**:** 13b and 13e) impaired promastigote growth and decreased the infection rate of peritoneal macrophages. Treatment with 13b and 13e alone modulated the cytokine secretion of infected macrophages, with increased levels of IL-12 and TNF-α. Furthermore, the decreased infection rate in the presence of 13b correlated with increased NO production by macrophages. These results suggested that 13b and 13e are potential anti-leishmanial lead compounds with low toxicity against host cells and selective antiparasitic effects[93]. Similar results were obtained in another study conducted in Germany as the data obtained showed that both compounds targeted leishmanial cathepsin B-like cysteine catheps and induced promastigotes cell death[94]. Moreover, the anti-leishmanial activity of MDL 28170 (a potent calpain inhibitor) on the growth of *L. amazonensis* was evaluated. The inhibitor promoted cellular morphological alterations[95].

Two recombinant cystatins (HvCPI5 and HvCPI6) were tested *in vitro* against promastigotes and intracellular amastigotes of *L. infantum* in the J774 monocytic cell line. Low concentrations from both cystatins were unable to inhibit promastigote replication, while HvCPI5 was toxic for mammalian cells. Results pointed towards the direct inhibition of amastigote multiplication by HvCPI6 and the interest in this recombinant cystatin for leishmaniasis chemotherapy[96].

***Trypanosoma* species**

A CP (Trypanopain-Tc) with cathepsin-L-like properties has been purified from *T. congolense*. This CP was inhibited by cystatin, E-64 and a variety of peptidyl diazomethanes[97]. In 2000, a study conducted in South Africa showed that chalcones, acyl hydrazides and amides killed cultured *T. b. brucei*, through inhibition of trypanopain-Tb (the major *T. b. brucei* CP)[98]. Diazomethane inhibitor was recognized to kill *T. brucei in vitro* in another study conducted in 2004[99].

Moreover, one of the VS compounds (K11777) which is considered an irreversible inhibitor of cathepsin L-like CPs was expressed at higher levels by *T. b. gambiense*. These *in vitro* studies implicated brucipain as a critical driver of *T. b. gambiense* trans-endothelial migration of the human blood brain barrier (BBB)[100]. Recently, it was shown that brucipain stimulated G protein coupled receptors that lead to the activation of Galpha(q)-mediated calcium signaling. The consequence of these events was predicted to increase BBB permeability to parasite transmigration and the initiation of neuroinflammation events precursory to CNS disease[101].

T. cruzi invasion to non-phagocytic cells was impaired by membrane-permeable CPIs such as Z-(SBz)Cys-Phe-CHN(2) but not by cystatin C. It was shown that invasion competence is linked to the kinin releasing activity of cruzipain. Therefore, the investigators proposed cruzipain as a Chagas' disease virulence factor[102]. On the other hand, the intersection of chagasin, an endogenous CPI in *T. cruzi*, and cruzipain trafficking pathways may represent a checkpoint for downstream regulation of proteolysis in trypanosomiasis[103]. In addition, it was suggested that chagasin regulates CP endogenous activity, thus indirectly modulating proteolytic functions essential for parasite differentiation and mammalian cells invasion[104]. In 2008, a study was conducted in USA, with the goal of developing potent non-peptidic cruzain inhibitors. The investigators searched in the substrate activity screen library for protease substrates initially designed to target the homologous human protease cathepsin S. It was found that one of VS compounds completely eradicated *T. cruzi* from mammalian cell cultures and consequently has the potential to lead to new chemotherapy for Chagas disease[105]. Two years later, the same investigators established a non-peptidic tetra-fluoro-phenoxy-methyl ketone cruzain inhibitor

which ameliorated symptoms of acute Chagas disease in a mouse model with no apparent toxicity[106].

Both *T. cruzi* and *T. brucei*; the causative agents of American and African trypanosomiasis, rely on essential CPs for survival; cruzain and rhodesain, respectively, that are known previously as trypanopains. In 2010, another study conducted in USA identified triazine nitriles as promising drug targets for treatment of both American and African trypanosomiasis[107]. Recently, other investigators designed, synthesized and evaluated a series of azanitrile-containing compounds, most of which were shown to potently inhibit both recombinant cruzain and rhodesain. Results showed that these compounds have great promise as a new class of anti-trypanosome agents[108].

An article in the literature review dealing with *in vitro* inhibition of transformation of *T. cruzi* trypomastigotes into amastigote, and *vise versa* using LC, documented the essential role of proteasomes in *T. cruzi* stage-specific transformation[109].

***Schistosoma* species**

A proteolytic enzyme (SM32) was shown to be a developmentally regulated enzyme for schistosomula survival. Using a specific enzyme inhibitor *in vitro* resulted in death of 75% of the schistosomulae[110]. In another study carried in USA, two distinct types of irreversible CPIs were evaluated in *Schistosoma*-infected mice. The results showed a significant reduction in worm burden, hepatomegaly and egg number produced/female worm. Histopathology showed a minimal immune response to the produced eggs, consistent with a delay in egg production relative to untreated infected mice[111]. On the other hand, aza-peptide epoxides are irreversible CPIs that had little or no inhibitory activity with other proteases such as caspases, chymotrypsin, papain, cathepsin B, granzyme B, and various aspartyl proteases[112].

Adult *S. mansoni* utilizes host hemoglobin as a nutrient source, and its cathepsin B (SmCB1) was thought to have a central role in its hemoglobin digestion. However, results of a study conducted in 2005 indicated that SmCB1 is necessary for normal parasite growth[113]. Moreover, the efficacy of the VS compound (K11777) which targeted SmCB1 was evaluated in the murine model of *S. mansoni*, and it cured parasitologically 5 out of 7 infected mice, reduced worm and egg burdens, and ameliorated organ pathology. The investigators concluded that this CPI validates schistosome CPs as drug targets and offers the potential of a new direction for chemotherapy of human schistosomiasis[114].

On the other hand, it was shown that the aspartic protease cathepsin D is expressed in the schistosome gut where it plays an apical role in digestion[115], while *S. mansoni* cathepsin L3 (SmCL3) may contribute to the network of proteases involved in degrading host blood proteins as nutrients[116]. Cystatins play a crucial role in the immune evasion from their host and in the adaptation to host defense[117].

***Fasciola* species**

It was shown that 11 proteases were released by immature and mature *F. hepatica* flukes, their activities were inhibited by leupeptin, L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane, phenyl-methyl-sulfonyl fluoride and iodoacetamide[118]. On the other hand, the juvenile flukes release papain or cathepsin B CP which degrades hemoglobin and collagen[119], and cleaves immunoglobulins (Igs)[120]. It was found that peptidyl diazomethyl ketone labeled with a 2,4-dinitrophenyl (DNP) acted as a good inhibitor for bovine cathepsin B, human cathepsin L and *F. hepatica* cathepsin L-like protease (FheCL)[121]. In 2008, VS compounds were found to show potent activity against *F. gigantica in vitro* causing immediate death of adult flukes[122].

A study carried out in Ireland showed that FheCL suppressed IFN-gamma production which was attenuated in IL-4 defective mice. The suppressive effect was abolished by CPI (Z-Phe-Ala-diazo-methyl-ketone), i.e. FheCL was involved in Th1 immune responses suppression which depends upon IL-4[123]. Recently, another study conducted in Ireland showed that fluorobenzoyl dipeptidyl derivatives could be used as inhibitors of FheCL1[124].

Three studies were conducted in Mexico by the same investigators. Results of the 1st study showed that E-64 reduced 85% of *F. hepatica* CP proteolytic activity in the liver of cathepsin B knockout mice[125]. In the 2nd study, they showed that E-64 not only inhibited liver proteolytic activity but also produced anti-fecundity and anti-embryonation effects, delaying the progression of fascioliasis[126]. In 2007, CPI (Ep-475) reduced liver damage with impairment of liver fluke growth and fecundity. These findings pointed at liver fluke proteases as potential targets for pharmacological intervention[127].

On the other hand, cystatins, a potent FheCL inhibitor was found to modulate host immune responses[128]; while FhCB1 showed resistance to inhibition by cystatin family inhibitors from sheep and humans. It appears that FhCB1 protease functions largely as a digestive enzyme in the parasite gut[129]. Similarly, type 1 cystatin**,** a major antigen released by *F. gigantica* (FgStefin-1), had protective functions regulating intracellular CP activity and protecting against extracellular proteolytic damage to the parasite's intestinal and tegumental surface proteins[130].

Onchocerca volvulus

Cathepsin B-like CP was shown to be present in the hypodermis and cuticle of L3 and female *O. volvulus*, and in the egg shell around developing microfilariae[131]. The same investigators found that onchocystatin (OV7) is an active CPI as it inhibited 50% of the enzymatic activity of the bovine CP cathepsin B[132]. Moreover, Cathepsin B was detected as an excretory-secretory (E/S) product of the microfilariae suggesting a possible role of onchocystatin in the parasite evasion to vector immune response[133]. On the other hand, a study conducted in Germany showed that recombinant onchocystatin (rOv17) has modulatory effects on human T cell responses and macrophage functions which contribute to a state of cellular hypo-responsiveness and a possible pathogenicity factor essential for the persistence of *O. volvulus* within its human host[134]. Moreover, culturing L3 *in vitro* in presence of anti-onchocystatin human Ig and naïve neutrophils resulted in inhibition of molting process and larval cytotoxicity[135].

In 2002, Hartmann *et al*.[136] showed that cystatins were potent triggers of the production of NO, a mediator with essential role as an effector molecule against *O. volvulus in vitro* and *in vivo*. A year later, another study showed that *O. volvulus* cystatins down-regulated proliferative responses of T-host cells which reflected *O. volvulus* adaptation to their parasitic life style[137].

Other filarial worms

A 17-kDa antigen (Av17) of the rodent filarial parasite *Acanthocheilonema viteae* with amino acid homologies to cystatin C was released by filariae *in vitro*. It markedly suppressed mitogen-induced T cell proliferation of mice and induced down-regulation of murine T cell responses to mitogens, T cell receptor cross-linking using anti-CD3 antibodies, and also to specific antigens through IL-10 time up-regulation. So, cystatin C was considered an effector immunomodulation molecule and a potential target for anti-filarial intervention[138]. On the other hand, a 15-kDa protein located on the surface of both L3 and adult *B. malayi* was reported as a member of the cystatin. This molecule (Bm-CPI-2) blocked conventional CPs such as papain involved in the Class II antigen processing pathway in human B cells[139]. An *in vivo* study conducted in Germany showed that cystatins reduce NO production upon microfilarial challenge. Furthermore, antigen-specific proliferative response of spleen cells to circulating *Litomosoides sigmodontis* microfilariae was significantly diminished. These results suggested that cystatin acts as an immunomodulatory molecule during the course of a filarial infection, and its neutralization might contribute to generate protective immune responses[140].

On the other hand, targeting apoptotic signaling pathway and pro-inflammatory cytokine expression using calpain inhibitors (ALLN) as therapeutic intervention in tropical pulmonary eosinophilia induced lung damage[141]. Finally, a cathepsin L-like CP detected in ES products of *Brugia pahangi* molting L3 was found to have an essential role in its transformation to L4[142].

The following table summarizes the non-specific CPIs that were used in the literature for different parasitic diseases.

Concluding Remarks

1. Almost all parasitic CPs play a vital role in parasite nutrition, host cell invasion and host immune response evasion. Therefore, inhibitors of these proteases are promising chemotherapeutic targets for parasitic infections.
2. Three CPs (EhCP1, EhCP2 and EhCP5) are responsible for approximately 90% of the CPs activity in *E. histolytica*. Venyl sulfons compounds, pan caspase inhibitor and chagasin-like inhibitor family may be useful candidates with anti-amoebicidal effects.
3. Falstatin is a crucial CP in malignant malaria and treatment of late schizonts with antibodies that blocked the inhibitory activity of falstatin decreased the subsequent invasion of erythrocytes by merozoites. In addition, chagasin and brucipain are essential CPs for regulation of endogenous CP activity, and parasite differentiation and host cells invasion in American and African trypanosomiasis, respectively.
4. Cathepsins (L and B) are targets for development of chemotherapeutic agents in several parasitic diseases. They are found in all *Leishmania* species and are required for parasite growth and/or virulence. Inhibition of these proteases was achieved with both reversible and irreversible inhibitors. In addition, PVS derivatives impaired *T. gondii* host invasion and gliding motility through their effects on the parasite's cathepsins. Onchocystatin (OV7) is an active CPI as it inhibited the enzymatic activity of cathepsin B detected as an excretory-secretory product of *O. volvoulus* microfilariae.
5. The role of CPIs on host immune responses was evaluated in several parasites. In *L. mexicana* infections, inhibition of cathepsin B-like proved to be a valuable immunomodulatory strategy for chronic forms of leishmaniasis. Cystatins also play a crucial role in modulation and/or evasion of the host immune response in schistosomiasis and fascioliasis. On the other hand, cysteine and serine proteinase inhibitors reduced *T. vaginalis* ability to cleave immunoglobulins.

CP Inhibitor	Parasite	Function	Ref.
ALLN	*P. falciparum*	Inhibitory effects on the erythrocytic stages of *P. falciparum*	74
	Filarial *spp.*	Targeting apoptotic signaling pathway and pro-inflammatory cytokine expression	141
Antipain	*Cryptosporidium spp.*	Reduction of 10-15% in parasite number *in vitro*	35
	T. vaginalis	Immunoglobulins cleavage	44
	L. donovani	Role of apoptosis in leishmaniasis	90
Aprotinin	*Cryptosporidium spp.*	Reduction of 10-15% in parasite number *in vitro*	35
Aza-peptide epoxides	*Schistosoma spp.*	Little inhibitory activities on caspases, chymotrypsin, papain, cathepsin B, granzyme B & various aspartyl proteases	112
Aziridine-2,3-dicarboxylates	*Leishmania spp.*	Modulation of cytokine secretion of infected macrophages, with increased levels of IL-12 and TNF-α.	93
BDA-410	*P. chabaudi*	Inhibition of parasite growth *in vitro* and delay in the malaria progression *in vivo*	77
Diazomethane	*T. brucei*	Killing the parasite *in vitro*	99
E-64	*G. lamblia*	Inhibition of parasite adhesion to epithelial cells	31
	G. lamblia	Inability of cyst to excyst	32
	Cryptosporidium spp.	Inhibition of azocasein proteinase on the sporozoites surface	33
	T. vaginalis	Immunoglobulins cleavage	44
	T. vaginalis	Inhibition of a proteinase which degraded collagens I, III, IV and V, human fibronectin hemoglobin and IgA and IgG.	45
	Plasmodium spp.	Prevention of RBCs rupture, alteration of schizont morphology	67
	Plasmodium spp.	Inhibition of cell invasion *in vitro*, and sporozoite infectivity *in vivo*	68
	P. falciparum	Inhibition of oocyst production	69
	L. mexicana	Ablation of the CP to induce IgE response	81
	T. congolense	Killing the parasite	97
	F. hepatica	Reduction of 85% of proteolytic activity of livers of infected mice.	125
		Anti-fecundity & anti-embryonation effects delaying progression of fascioliasis	126
E-64 & PMSF	*Cryptosporidium spp.*	Inhibition of azocasein hydrolysis	34
EDTA	*Cryptosporidium spp.*	Inhibition of azocasein proteinase on the sporozoites surface	33
EGTA-AM	*Plasmodium spp.*	Prevention of RBCs rupture, alteration of schizont morphology	67
Ep-475	*F. hepatica*	Reduction of liver damage with impairment of adult fluke growth and fecundity	127
Hydrogen peroxide	*Cryptosporidium spp.*	Inhibition of the protease activity up to 50%	36
K11777	*E. histolytica*	Inhibition of amoebic invasion in human colonic model	20
	T. b. gambiense	Inhibition of cathepsin L-like to induce trans-endothelial migration of the human blood brain barrier	100
	S. mansoni	Cure of infected mice (reduction of worm & egg burden)	114
Iodoacetamide	*T. vaginalis*	Immunoglobulins cleavage	44
	Fasciola spp.	Inhibition of the activity of 11 proteases released by immature and mature flukes	118
Iodoacetic acid	*Cryptosporidium spp.*	Inhibition of azocasein proteinase on the sporozoites surface	33
	T. vaginalis	Immunoglobulins cleavage	44
Leupeptin	*Cryptosporidium spp.*	Reduction of 40-50% in parasite number *in vitro*	35
	T. vaginalis	Diminished ability to bind to epithelial cells with inability to kill host cells	42
	L. donovani	Role of apoptosis in leishmaniasis	90
	Fasciola spp.	Inhibition of the activity of 11 proteases released by immature and mature flukes	118
Peptidyl diazomethyl ketone	*F. hepatica*	Abolishment of the suppressive effects induced by FheCL on IFN-γ.	121
PMSF	*Cryptosporidium spp.*	Reduction of 40-50% in parasite number *in vitro*	35
	Fasciola spp.	Inhibition of the activity of 11 proteases released by immature and mature flukes	118
Phosphoramidon	*Cryptosporidium spp.*	Inhibition of azocasein proteinase on the sporozoites surface	33
SBTI	*Cryptosporidium spp.*	Reduction of 40-50% in parasite number *in vitro*	35
TFPM	*T. cruzi*	Amelioration of symptoms of acute Chagas disease in a mouse model.	106
TLCK	*T. vaginalis*	Diminished ability to bind to epithelial cells with inability to kill host cells	42
	T. vaginalis	Immunoglobulins cleavage	44
	L. donovani	Role of apoptosis in leishmaniasis	90
TPCK	*G. lamblia*	Inhibition of parasite adhesion to epithelial cells	31
	T. vaginalis	Diminished ability to bind to epithelial cells with inability to kill host cells	42
	T. gondii	Breakdown of the parasite surface membrane	53
VS compounds	*P. falciparum*	Inhibition of the CP	63,64
	P. vivax	Inhibition of CPs	76
	F. gigantica	Potent activity causing immediate death of the adult flukes	122
WRR483	*E. histolytica*	Inhibition of amoebic invasion in human colonic model	20
WRR605	*E. histolytica*	Reduction of the parasite burden and inflammation in the mouse cecal model.	21
Z-Phe-Ala-diazo-methyl-ketone	*F. hepatica*	Abolishment of the suppressive effects induced by FheCL on IFN-γ.	123
Z-SBz-Cys-Phe-CHN(2)	*T. cruzi*	Impaired invasion to non-phagocytic cell	102

***PMSF:** Phenyl-methyl-sulfonyl fluoride, **SBTI:** Soybean trypsin inhibitor, **TFPM:** Tetra-fluoro-phenoxy-methyl ketone

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