ABSTRACT

Expression of cysteine proteinases and cystatins in parasites and use of cysteine proteinase inhibitors in parasitic diseases.

Part III: Protozoa (1)

Sherif M Abaza

Medical Parasitology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Out of five classes of proteases (cysteine, serine, threonine, aspartate and glutamate), cysteine proteases (CPs) are responsible for hydrolysis of peptide bonds essential in several biological activities. In protozoa, as with helminths, not only do CPs play the major role in nutrients digestion, but they also have several functions for parasite survival such as differentiation of life cycle stages, immunomodulation of host immune response, and autophagy. Most well-characterized CPs in protozoa that were investigated in the last two decades belong to papain-family enzymes (clan CA, family C1). The present review highlights, in general, several aspects of CPs functions in protozoal survival and developmental stages, essential functions, as well as genomic organization. They also discussed the feasibility of application of these CPs in development of vaccine candidates.

Furthermore, CPs were utilized as diagnostic markers. In 2005, Brazilian investigators described SDS-PAGE for in situ detection of CP activity using its specific CP inhibitor to differentiate between flagellates isolated from insects and plants. In the same year, a group of scientists from the UK employed genomic analysis to identify genes encoding CALPs in three human kinetoplastids: L. major, T. cruzi, and T. brucei. They

Cysteine proteinases (CPs)

Being essential for parasite survival, CPs are investigated for development of vaccine candidates and/or novel drugs in protozoal diseases with high mortality and morbidity rates such as malignant malaria, African sleeping sickness and Chagas' disease, as well as neglected tropical diseases such as visceral leishmaniasis, toxoplasmosis, extraintestinal amoebiasis and cryptosporidiosis. Caffrey and Steverding[1], in their review, introduced a unified nomenclature for kinetoplastid cathepsins (CATHs) and discussed their expressions in different developmental stages, essential functions, as well as genomic organization. They also discussed the feasibility of application of these CPs in development of vaccine candidates.

Recent evolution in molecular technology and bioinformatics enable researchers to localize and analyze the biologic cellular functions of several expressed CPs. In addition, CPs abundance in parasites compared to their mammalian hosts attract scientists to search for specific CP inhibitors for development of safe chemotherapeutic agents without host adverse side effects. In this regard, Caffrey et al.[2] reviewed CPs of clan CA, family C1 in kinetoplastids and discussed their biochemical and genetic diversity as well as their genomics in relation to stage-specificity and expression control. The reviewers aimed at utilizing literature date as first step to achieve development of novel drugs and/or vaccine candidates to control visceral leishmaniasis, African sleeping sickness and Chagas' disease. On the other hand, complete genome sequences of three human pathogenic trypanosomatids, T. brucei, T. cruzi and Leishmania spp. followed by in silico analyses allowed the researchers to identify and assign 23, 40 and 33 genes involved in calpains (CALPs) expression in different stages of T. brucei, T. cruzi and L. braziliensis, respectively. In a review article published two years later, the reviewers discussed the biochemical and biological aspects of CALPs expressed in these trypanosomatids. They focused on the fact that knowing the genome sequences in drug-resistant and -sensitive strains will allow the investigators to investigate the inhibitory efficiency of several CALP inhibitors[3].
succeeded to define 27, 24 and 18 genes, respectively. Amino acid sequences of the defined genes revealed high modular structure, suggesting the feasibility to utilize specific primers as diagnostic markers[9].

Recently, Siqueira-Neto et al.[6] reviewed the proposed functions of the most characterized 29 CPs only in seven protozoa; E. histolytica (six), Leishmania spp. (six), Plasmodium spp. (five), T. gondii (five), T. cruzi (three), T. brucei (two), and Cryptosporidium spp. (two). It is evident that the most common proposed character of these CPs is a virulence factor to facilitate parasite survival and invasion. For each CP, the reviewers presented the mechanism(s) to achieve parasite invasion including induction of macrophage pro-inflammatory response, degradation of extracellular matrix, differentiation of life cycle stages, modulation of parasite metabolism, and autophagy. Mechanisms involved in immunoevasion and immunomodulation of host immune response are also proposed in all reviewed protozoa. There are other proposed mechanisms specified for some protozoa such as encystation-excystation transformation, and degradation of host IgA and IgG (E. histolytica), crossing blood brain barrier (T. brucei), hemoglobin degradation, enhancement of oocysts production, sporozoites invasion of hepatocytes, and apicoplast development and homeostasis (Plasmodium spp.), and high expression in tachyzoites for digestion of cytolic proteins (T. gondii).

Beside their role in parasite invasion, CPs of apicomplexan protozoa are required for pathogen exit from the infected cells to invade other cells and continue the infection. In Plasmodium spp. and T. gondii, being obligate intracellular pathogens, schizogony or endodyogeny, involve replication within a specialized parasitophorous vacuole (PV) to yield multiple merozoites or tachyzoites, respectively. Both host calcium and CALP-1 are implicated in rupture of the infected cells, while apicomplexan CALPs are implicated in escape of merozoites and tachyzoites from the PV by their proteolysis-dependent mechanism[9]. In another report published in 2009, the investigators discussed the role of CALP of apicomplexans Plasmodium spp. and T. gondii in parasite egress. They showed that in vitro addition of DCG04 (a derivative of nonspecific papain family protease inhibitor E6-4) to Plasmodium-infected erythrocytes revealed blocking in schizont-stage and trapping of merozoites in PV within intact red blood cell membranes. Infected RBCs were treated with saponin to dissolve PV membranes, then centrifuged to remove parasite cells, and pelleted to produce purified soluble fraction. Mass spectrometry identified only host CALP-1, confirming its involvement in RBCs rupture. When CALP-1 depleted erythrocytes were treated with DCG04, parasite kinetics was improved to some extent, suggesting the importance of apicomplexan CALPs in parasite egress. The investigators concluded that both CALPs of Plasmodium spp. and T. gondii exploit host cell CALPs to facilitate escape from PV or host plasma membrane, but they failed to explore the precise mechanism[9].

Apoptosis
Apoptosis is an essential host pathway contributing both innate and acquired immune responses. It can be induced via either intrinsic or extrinsic pathways. The first is stimulated by cellular stress signals such as DNA damage, lack of essential growth factors, or infection. The extrinsic pathways are activated via death receptor ligation mechanism used by cytotoxic cells (T, natural killer, and non-lymphoid cells) to induce cell death[9]. It was reported that some cytotoxic cells can induce cell death via the perforin-dependent granule exocytosis pathway[10]. In intracellular pathogens such as viruses, bacteria and protozoa, host cytotoxicity plays an important role to establish efficient immune defense mechanism(s). On the other hand, intracellular pathogens must interfere with cell apoptosis to protect their host cells, and themselves, from cell death[11].

In a review published in 2011[12], the British scientists claimed that apoptosis is an essential phenomenon for normal development and survival in multicellular parasites, whereas its occurrence in unicellular protozoa seems strange since they have to evolve strategies to increase their replication, not death; i.e., self-regulate the intensity of infection in the host or vector. The first question in their review was "Do protozoa commit suicide to survive?" First, they drew a diagram showing that cell death is either passive, due to extrinsic factors, leading to rapid irreversible necrosis with membrane disruption and damage of organelles; or active, due to intrinsic factors, leading to programmed cell death (PCD), involved in a regulated step-manner which can be reversible before the final stage is reached. PCD is either slow leading to autophagy or fast resulting in apoptosis. In autophagy, there is downregulation of metabolic processes with digestion of organisms, while in apoptosis, there is controlled cascade with morphological events and functional cell breakdown and eventually cell death. Markers of cell apoptosis include DNA fragmentation, chromatin condensation, membrane blebs, cell shrinking, proteins cleavage by proteolysis, and release of proteins from mitochondria. The second question was "how cell apoptosis with parasite number reduction can assist the survivors?" The answer was that it depends on density of parasites, and that logically, no gained benefits will be obtained for low parasite density survivors. In contrast, if the parasite number is high enough to cause host or vector survival at risk, the best strategy for unicellular protozoa is to undergo apoptosis to maintain a sub-lethal density, i.e. higher apoptotic parasite number leads to bigger benefits to survivors. The third question was "how parasites get information about low or high density? Or is it the time to commit suicide or to proliferate?" The answer is it doesn't matter to have this information, as natural
selection shapes parasite strategy usually in line with the parasite density. The reviewers pointed out that “one-size-fits-all” strategy is the least outcome when variation in parasite density is an achieved experience during infections in different hosts. However, more sophisticated strategies become possible in case of parasites ability to get that information; as with other parasite strategies, e.g., gene mutations attempted in drug resistance. Previous studies reported that *P. falciparum* [15] and *T. brucei* [14] can determine the genetic diversity of their infections suggesting its ability to estimate the density or proliferation rate of their clone-mates.

Because caspases are limited to metazoans, the first description of caspase orthologues was proposed as paracaspases from animals and metacaspases (MCAs) from unicellular pathogens such as fungi and protozoa [15]. All caspases and orthologues are clan CD, family C14, however, they show difference only in substrate specificity. MCAs, with their highly acidic S1 pocket, have high basic specificity for arginine and lysine at the P1 position, rather than aspartic acid specificity for caspasps [16]. Two types of MCAs are known, however, only type I was detected in protozoa. It is characterized by having a N-terminal prodomain, while type II MCAs have a linkage between the p20 and p10 domains instead [17].

It is worth mentioning that some, not all, of apoptotic markers were observed in unicellular protozoa such as *T. cruzi*, *P. berghei*, *Leishmania* spp., *Blastocystis* spp., and *T. vaginalis* [18-22]. On the other hand, MCAs role in parasite apoptosis was investigated in *T. cruzi*, *L. major*, and *T. gondii* [23-25].

Cysteine proteinase inhibitors (CPIs)

Several strategies are employed to identify or synthesize safe and potent CPs inhibitory compounds. Virtual screening of 241 thousand compounds in ChemBridge database identified 24 CP inhibitors (CPIs), among them four compounds showed efficient CPs inhibition of *P. falciparum* and *L. donovani* [26]. Screening a library including synthesized thio-semicarbazones identified several promising leading compounds that showed high activity against falcipain-2, rhodesain and cruzain; the major CPs in *P. falciparum, T. brucei* and *T. cruzi*, respectively. In addition, their toxicity was tested in mice and only one compound showed observable toxicity after 62 h. The investigators recommended further studies to validate use of thio-semicarbazones as CPIs [27]. Several gold compounds were investigated against CATH L-like and CATH B-like; the major CPs of *T. brucei rhodesiense* and *L. mexicana*, respectively. According to the promising results, some gold compounds showed potent inhibitory efficiency with IC50 in the micromolar concentrations. When these compounds were investigated against the growth of the causative protozoa, the investigators observed lack of correlation between *in vitro* assays of both CPs inhibition and antiprotozoal activities [28]. Recently, compounds synthesized from screened dipeptidyl enoates were also investigated against falcipain-2, rhodesain, and cruzain. The investigators also investigated their selectivity against human CATHs B and L1. Their results showed that they were potent irreversible CPIs, and *in vivo* antiprotozoal studies showed promising results for treatment of malignant malaria, African sleeping sickness and Chagas’ disease, with IC50 values in the micromolar range [29].

Another strategy is based on the fact that compounds supplementing the host with nitrous oxide (NO-donors) inactivate CPs. Italian investigators published two articles to show the effects of several NO-donors; dansyl-SNO, GSNO, NOR-3, SNAP [29], and SNO-102 [30] on CP activity of *P. falciparum*, *T. cruzi* and *L. infantum*. Results revealed that NO-donors are efficient CPIs, in a dose- and time-dependent manner. Similarly is the use of Michael acceptors to inactivate CPs through addition of Michael donors to combine with CP, forming a double stabilized carbon nucleophile. In this regard, two reports were published. The first study was conducted in Brazil and described synthesis of novel dipeptidyl alpha-fluoro-vinyl sulfones. The results showed that addition of Michael receptor to synthesized compounds revealed satisfactory inhibition of *L. mexicana* CP [30]. The second German study reported that diastereomeric E-configured vinyllogous dipeptide esters were the most active inhibitors against the major CPs of *P. falciparum* (falcipain) and *T. brucei* (rhodesain). However, whereas maleic acid derivatives displayed satisfactory inhibition only against rhodesain with efficient anti-parasite activity, fumaric acid derivatives displayed inhibition of both CPs without anti-parasitic activity [31].

Utilizing compounds from herbs and coral as a strategy for discovery of CPIs, garlic allicin derivatives were reported to inhibit falcipain 2, rhodesain and CATHs B and L, with potent *in vitro* anti-parasitic activity. German investigators observed that only derivatives with primary carbon atom were attacked by thiosulfinate sulfur atom of CP active-site Cys residue [32]. From the Caribbean coral Plexaura homomalla, Argentinian investigators isolated a fraction with tight-binding inhibitory effects against papain-like CPs. Results showed promising *in vitro* anti-parasitic effects against *T. cruzi* and *P. falciparum*, evident by growth reduction in resistant strains [33].

Finally, in their review, Siqueira-Neto and his colleagues [34] clarified that several CPIs were investigated, among them, only K11777 proved efficient inhibition of CPs of *T. cruzi*, *T. brucei*, *C. parvum*, *T. gondii*, *E. histolytica*, and *L. tropica* and is currently moving forward to clinical trials. Other CPIs under investigations include WRR-483, Cz2007, Cz2008 and Tetra-fluoro-phenoxy-methyl ketones for treatment of Chagas’ disease, and Gallinamide A and Symplostatin-4 for treatment of malignant malaria.
Cystatins (CYSs)

Protozoal cystatins (CYSs) are endogenous tight-binding reversible CPIs, categorized into three types (I, II and III) according to their molecular weight. As previously reviewed, CYSs play a major role in regulation of CPs expression in parasites, inactivation of host CPs, and immunomodulation of host immune response. Trials to develop novel drugs and/or vaccine candidates utilizing filarial CYSs, and utilizing hard tick CYSs as a measure for vector control strategy were previously reviewed.[36,37]. In protozoa, few CYSs were reported in T. cruzi (chagasin), L. mexicana, E. histolytica (EhCP), P. falciparum (falstatin), T. brucei (TbMCP), T. gondii (toxostatins), C. parvum (cryptstatin), Acanthamoeba spp. (AcStefin) and T. vaginalis (trichocystatins 1-3).[38-47]. All are considered virulence factors due to their major role in CPs-mediated pathogenesis in their respective diseases (will be reviewed later).

Toxoplasma gondii

Toxoplasma genome project revealed a much lower number of CP genes in T. gondii compared to other studied protozoal genomes. Only five genes encoding papain family CATHs were characterized from T. gondii, including one CATH B (TgCPB), one CATH L (TgCPL), and three CATH Cs (TgCP1-3). Dou and Carruthers[40] reviewed and summarized T. gondii CATHs. As T. gondii lacks specialized motility appendages, the reviewers attributed its ability to invade host cells to two factors; intracellular actin-myosin system to slide on the host cell surface, and proteins expressed by the dense organelles; micronemes (MICs), rhoptries (ROPs) and the vacular compartment (VAC). Genomic analysis of these genes showed that they are distributed among four distinct chromosomes. Chromosomes Ib, III and XII were identified for genes encoding CATHs L, C2 and B, respectively, while chromosome IX was whoch also. [51] etc.

Expression of both TgCPL and TgCPB in bradyzoites was also identified as proteolytic activities that trigger the turnover of autophagosomes (autophagy) and parasite persistence during neural chronic toxoplasmosis. The investigators observed that death of bradyzoites lacking VAC proteases was preceded by accumulation of undigested autophagosomes in VAC. Accordingly, the investigators concluded that in chronically infected mice, there is upregulation of TgCPL and TgCPB expression for VAC proteolysis favorable for longer persistence of the parasite.[52] Later, a similar conclusion was obtained both in vitro and in vivo.[53]
Cathepsin B: Toxopain-1 was the first identified \textit{T. gondii} CP and was shown to be localized in ROPs that play a crucial role in PV establishment; the first step in \textit{T. gondii} invasion process. The investigators succeeded to clone the full-length of the gene encoding toxopain-1 \textit{(tcp1)} and the resultant amino acid sequence was \sim50\% identical to human liver lysosomal CATH B. It was demonstrated that the identified toxopain-1 \textit{(TgCPB)} is the key enzyme for ROP proteins processing, as the use of specific CPIs led to \>60\% inhibition of processing of ROP2, 3, and 4. Therefore, Que et al.\cite{56} recommended the use of specific CPIs as novel drug targets to prevent and/or treat toxoplosmosis. Two years later, the same investigators observed that \textit{TgCPB} decreased expression resulted in \textit{in vitro} inhibition of tachyzoites growth. To conduct \textit{in vivo} studies, the investigators modified a chicken embryo model to evaluate effects of intravenous inoculation of tachyzoites on size of embryo. This was followed by addition of specific \textit{TgCPB} inhibitors that decreased level of infection with \>80\% decrease in the number of brain and hepatic tachyzoites\cite{58}. In a study conducted in USA\cite{58}, two important observations were elucidated. One was that \textit{TgCPB} is co-localized with \textit{TgCPL} in VAC, instead of ROPs. Using RH \textit{T. gondii} as well as its different mutant strains, the investigators were able to achieve definite localization and dynamic maturation of \textit{TgCPB} utilizing immunofluorescence assays and immunoelectron microscopy. The other was that \textit{TgCPB} expression is neither required for maturation of \textit{TgROPs} 2, 3 and 4 nor for ROP biogenesis.

**Cathepsin L:** Guided by \textit{Toxoplasma} cDNA library, gene encoding \textit{TgCPL} was isolated, cloned and characterized. Two differences were detected between \textit{TgCPB} and human CPL, its optimum acidic pH and its preference to substrate with leucine in the P2 position, instead of phenylalanine\cite{59}. In the same manner as \textit{TgCPB}, \textit{TgCPL} was found to have crucial role in host cell invasion through the apically stored adhesins; MIC proteins are among them. They are transmembrane proteins that bind to accessory proteins known as MIC-associated proteins (MAPs) to form MIC-MAP complex that is involved in host cell attachment and invasion. \textit{TgCPL} contributes to the proteolytic maturation of pro-\textit{TgM2AP} and pro-\textit{TgMIC3}\cite{60}. Later, Dou and his colleagues\cite{61} showed the contribution of \textit{TgCPL} expression in \textit{TgCPB} maturation in intracellular replicated tachyzoites, hence its activation. Their results demonstrated the profile of \textit{TgCPB} proteolytic activity and the cleavage sites of \textit{TgCPL} in pro-\textit{TgCPB}. Also, the investigators observed that VAC was fragmented into at least two distinct vesicles marked with both CATHs, however, \textit{TgCPL} alone was localized in distinct vesicles, while \textit{TgCPB} was not localized without \textit{TgCPL}\cite{62}.

On the other hand, it was documented that \textit{TgCPL} has a major role in utilizing host cytosolic proteins during intracellular replications. Several hypotheses were previously suggested. First, small pores in PV membrane were antagonized by trafficking of cytosolic macromolecules. Second, the suggested role of endoplasmic reticulum membrane to fuse with PV membrane to deliver parasite antigens to MHC class I pathway, and a similar mechanism was suggested for macromolecules. Third, the role of dense granules secreted by tachyzoites in PV. To investigate these hypotheses, a team of scientists from USA and Italy\cite{63} observed similar profiles in ingestion and digestion of host-derived proteins in both virulent type I and avirulent type II strains. They demonstrated tachyzoites ability to internalize cytosolic proteins through intravascular network arising from VAC. Reduced ingestion was observed in mutant tachyzoites lacking intravascular network, suggesting its implication as a transporter for parasite trafficking. Finally, the study showed that host macromolecules were accumulated in tachyzoites endolysosomal system of strains with mutant deficiency of \textit{TgCPL} expression or activity\cite{64}.

Besides its role in tachyzoites invasion and replication, activation of \textit{TgCPB} and digestion of host cytosolic proteins, \textit{TgCPL} proved to play an important role for cyst survival in chronic toxoplosmosis\cite{65}. Microscopic examination of cysts either incubated with LHVS or \textit{TgCPL} mutant strain, showed that bradyzoites have large, dark cytoplasmic inclusions that were absent from wild or non-treated strain. Stage specific \textit{TgCPL} re-expression during cyst stage, eliminated these inclusions. Based on the obtained results, the investigators pointed out the vital role of \textit{TgCPL}, either through itself or its role in \textit{TgCPB} activation, in persistent host neural infection\cite{53}.

**Cathepsin C:** Whereas \textit{TgCPC1}, also known as dipeptidyl peptidase I (DPPI), showed the highest expression in tachyzoites, \textit{TgCPC3} was found to be expressed only in sporozoites. In contrast, none of them were detected in mature encysted bradyzoites isolated from brains of chronically infected mice, suggesting negative functions for bradyzoites growth. A specific CPI inhibited their activities and reduced \textit{T. gondii} intracellular growth and proliferation. The investigators showed that CATH Cs have a crucial role in peptide degradation in the PV allowing for successful establishment of parasite growth and differentiation\cite{66}.

**CATHs as vaccine candidates:** Using bioinformatics, prediction of immunogenic B and T cell epitopes is a recent powerful tool for vaccine development. The potentiality of using \textit{TgCPB} and \textit{TgCPL} as DNA vaccine against toxoplosmosis was investigated for the first time in a study conducted by Chinese investigators. The investigators identified 21 and 17 potential epitopes on \textit{TgCPB} and \textit{TgCPL}, respectively. Accordingly, they assembled DNA vaccines using single- and multi-gene constructs to immunize BALB/c mice against challenged toxoplosmosis. Although both single-gene constructs elicited strong humoral and cellular immune response, no complete protection was detected. However, multi-
gene construct gave the highest level of protection. The investigators recommended further studies to utilize both CATHs to control toxoplasmosis[60]. Four years later, TgCPC1 as vaccine candidate was investigated also in China. Results revealed significant increase of IL-2 and IFN-γ, while T. gondii-specific IgG and IgG2a antibodies were non significantly higher than controls. However, other parameters such as IgG1, IL-4, and IL-10 were not affected, compared to controls. These results suggested that TgCPC1 mainly resulted in a specific Th1-type immune response. When the immunized BALB/c mice were challenged to evaluate its protective efficacy, results showed significant prolonged survival time compared to controls[61].

Apoptosis

It was observed that T. gondii-infected host cells are protected from apoptosis which leads to tachyzoites accumulation in non-apoptotic cell population[62]. In an investigation conducted in Germany, pathways through which T. gondii tachyzoites control host apoptosis-regulating cascades were demonstrated and characterized. The investigators suggested that extracellular tachyzoites, before entering new host cells, released molecules to inhibit cytochrome c that activates host caspases 3 and 7[63]. Since then, several studies investigated the role of T. gondii in controlling host apoptotic cascades; inhibition of Fas/CD95-triggered cell death via caspase 8 degradation[64], inhibition of perforin-dependent granule exocytosis pathway used by cytotoxic cells[65], decrease of poly(ADP-ribose) polymerase-1 (PARP-1) expression which greatly facilitated identification of its main target, and its inhibition subsequently led to impairment of host apoptosis[66]. Lastly, the prevalent Chinese strain TgCtwh3, that has polymorphic features of both type I and type II Toxoplasma assemblages, showed in vitro significant increase of apoptosis level of C17.2 cultured cells compared to negative control; but significantly lower than that of RH strain. The investigators suggested that TgCtwh3 strain induced apoptosis of C17.2 cells via up-regulation of caspase-12[66].

In all the previous studies, it seems that T. gondii tachyzoites compete with host apoptosis cascade to prevent cell death in order to survive and intracellularly replicate. The first study that pointed out possession of an apoptotic machine, later intracellular replication. The first study that pointed out possession of an apoptotic machine, later

in vivo extracellular tachyzoites (wild type) as well as apoptosis markers. On the other hand, reduction of apoptotic markers and greater viability of TgMCA mutant tachyzoites were observed. Accordingly, the investigators concluded that TgMCA had an essential role in parasite apoptosis, but is not essential for invasion and proliferation[25].

Cysteine proteinase inhibitors (CPIs)

In the early 20th century, two publications reported use of proteasome inhibitors for protection or treatment of toxoplasmosis. Lactacystin, a specific inhibitor in eukaryotic cells, proteasome inhibitor 1, and MG-132 showed efficient inhibition of T. gondii growth and daughter cell budding, as well as DNA synthesis. However, they neither blocked parasite entry nor inhibited PV establishment[71]. Similar results were obtained using another specific inhibitor (gliotoxin), with marked reduction in replication, but parasite penetration of host was not affected[72].

Using specific CATH inhibitor III, extensive morphological alterations of T. gondii excretory pathway with disrupted ROB formation were observed[73]. Two peptidyl vinyl sulfone compounds, LHVS and ZL3VS, efficiently blocked tachyzoites invasion. The investigators suggested TgCPL as drug target, and its inhibition subsequently led to impairment in release of MIC proteins[74]. Two years later, the same group of investigators identified two properties in one previously tested vinyl sulfone compound (LHVS), which greatly facilitated identification of its main target as TgCPL. LHVS mechanism of action is to covalently modify the active site thiol of reactive CATH, and its structure allows synthesis of a functional fluorescent derivative to identify its target CP using fluorescence microscopy. Accordingly, the investigators confirmed their previous proposal that TgCPL is the primary target of CPI/LHVS[73].

To develop a potent novel drug to treat chronic toxoplasmosis dormant bradyzoites in CNS, American investigators modulated a known specific inhibitor of human CPL to be selective against TgCPL, i.e. they modified physiochemical properties of the human CPL inhibitor, and guided by structure-based design of TgCPL, they succeeded to obtain more favorable inhibition regarding metabolic stability and CNS entry. Based on the obtained results, the investigators concluded that their strategy was efficient in development of a novel drug validating TgCPL as a feasible target for the treatment of chronic toxoplasmosis[75]. Recently, K11777 (an irreversible vinyl sulfone CPI) in vitro inhibited both TgCPB and TgCPL with EC50 = 114 nM and 71 nM, respectively. The investigators also observed that K11777 significantly inhibited tachyzoites (RH strain) invasion of in vitro human fibroblasts and almost blocked their intracellular replication in vivo chick embryo egg model (71% and >99%, respectively). They attributed their
Herbs with known CPI-enriched fraction, were also investigated for treatment of toxoplasmosis. Brazilian investigators utilized a protein fraction extracted from *Jatropha curcas* seed cake to develop a novel drug against toxoplasmosis. The extracted protein, in different concentrations, was investigated *in vitro* against *T. gondii*-infected Vero cells, and showed significant reduction of infected cells, with no alteration in Vero cells morphology up to 3.0 mg/ml\(^2\)h.

**Cystatins (CYSs)**

In 2009, Huang and his colleagues\(^{[43]}\), succeeded in identifying two genes encoding two endogenous CysPs; toxostatin 1 and 2 with molecular weights 17 and 25 kDa, respectively. Both CYSs proved to inhibit *TgCPL* and *TgCPB* in the nanomolar range (IC\(_{50}\) = 24.0 nM and 31.4 nM, respectively). However, toxostatin-1 inhibited more efficiently human CPL than human CPB (IC\(_{50}\) = 9.9 nM vs 146.5 nM). Utilizing *T. gondii* genome database and BLAST and protein motif program, the investigators were able to clone and identify sequencing of the genes encoding *T. gondii* CYSs. It was found that expression levels of both CYSs differed in parasite developmental stages; while toxostatin-1 high expression was observed in all stages (tachyzoites, bradyzoites and sporozoites), toxostatin-2 was limited only to tachyzoites. In addition, the investigators demonstrated that CYS-1 overexpression was followed by >90% reduction in both CATH proteolytic activity, only in transfected tachyzoite lysates. However, its overexpression did not affect either tachyzoites invasion within 2 h, or replication within 24 h, confirming the fact that CYSs are endogenous regulators of their CPs\(^{[43]}\).

**Concluding remarks**

1. Majority of protozoal CPs belong to clan CA, family C1 "papain-like family". Their expressions proved to have essential roles in all metabolic processes that require proteolysis.
2. Beside considering them as virulence factors for development of vaccine candidates and novel drugs, they are utilized as diagnostic markers.
3. Pathways of apoptosis in unicellular protozoa were previously thought as parasite’s defense against host apoptosis-cascade because cell death means parasite death. Nowadays, protozoa proved to have apoptotic molecules (metacaspases), and it was found that the higher apoptotic parasite number, the bigger benefits to survivors.
4. A dynamic multi-vesicular compartment, VAC, was characterized in *T. gondii* tachyzoite apical region. It showed associated dynamic functions with late endosomes throughout each phase of tachyzoites replication.
5. Among the five cathepsins characterized in *T. gondii*, *TgCPL* has the major role in parasite survival, invasion and replication. It also contributes in the proteolytic maturation of *TgCPB* pro-*TgM2AP* and pro-*TgMIC3*, essential molecules for tachyzoites invasion and replication. In addition, *TgCPL* has an essential role for ingestion and digestion of host cytosolic proteins during intracellular replications through an intravascular network arising from VAC.
6. Both *TgCPL* and *TgCPB* are expressed in bradyzoites to contribute in autophagosomes turnover (autophagy) accumulated in VAC, i.e. cyst survival. Therefore, *TgCPL* and *TgCPB* expressions are upregulated resulting in persistent chronic neural toxoplasmosis.
7. Among the three *TgCPCs*, *TgCPC3* was found to be expressed only in sporozoites, and none of them was detected in mature encysted bradyzoites.
8. Metacaspases of *T. gondii* have an essential role in parasite apoptosis but are not essential for invasion and proliferation.
9. Several vinyl sulfone compounds such as K11177, LHVS and ZL3VS efficiently block tachyzoites invasion and replications in acute toxoplasmosis as well as kill dormant bradyzoites in chronic diseases.
10. Endogenous *T. gondii* CPI (cystatins) toxostatin 1 and 2 are also described. They down regulate the major *T. gondii* cathepsins (*TgCPL* and *TgCPB*) throughout different parasite developmental stages. Its overexpression did not affect either tachyzoites invasion within 2 h, or replication within 24 h.

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