

# Expression of cysteine proteinases and cystatins in parasites and use of cysteine proteinase inhibitors in parasitic diseases. Part III: Protozoa (3): Kinetoplastids

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## ABSTRACT

Kinetoplastids, or trypanosomatids are flagellate protozoa characterized by the presence of a kinetoplast composed of a DNA network of circular molecules, localized near the basal body. Cysteine proteinases (CPs) have attracted considerable attention in pathogenic trypanosomatids over the last two decades due to their essential roles in parasite' growth, transformation, proliferation, migration and invasion, as well as immunomodulation of host immune system. Because CPs are essential virulence factors during all stages of the infection process, a number of new strategies to obstruct trypanosomatid biological processes have emerged; one of them is focused on using CP inhibitors (CPIs). The objective of the present review is to highlight the molecular characterization and functions of CPs in pathogenic trypanosomatids. Sufficient knowledge aided with bioinformatics analysis can lead to efficient development of diagnostic and biogenetic markers, drug targets (potent CPIs), and vaccine candidates. The role of the unusual endogenous CPI (CYS) in trypanosomatids will also be discussed.

**Keywords:** brucipain, calpain, cathepsin-like, chagasin, cruzipain, drug target, trypanosomatids, vaccine candidate.

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**Abbreviations:** **ATG:** Autophagy-related gene; **CALP:** Calpain; **CATH:** Cathepsin; **CPB2.8ΔCTE:** CPB2.8 copy without extension of its C-terminus; **CL:** Cutaneous leishmaniasis; **CP:** Cysteine proteinase; **CPI:** Cysteine proteinase inhibitor; **CRZ:** Cruzain; **CTE:** C-terminus extension; **CYS:** Cystatin; **E-64:** A broad spectrum CPI; **HTS:** High throughput screening; **MCA:** Metacaspase; **PCD:** Programmed cell death; **SAR:** Structural activity relation; **VL:** Visceral leishmaniasis.

Cysteine proteinases (CPs) in pathogenic trypanosomatids are virulence factors, and promising targets for development of novel drugs and vaccine candidates<sup>[1-4]</sup>. The most frequently studied CPs belong to cathepsins (CATH; clan CA, family C1), calpains (CALP; clan CA, family C2) and metacaspases (MCA, clan CD, family C14). In all trypanosomatids, CATH-like CPs possess a long C-terminus extension (CTE). Cathepsin-like CPs with their CTE and their crucial roles as virulence factors and capability to elicit host immune response, as well as their applications as drug targets, diagnostic or biogenetic biomarkers and vaccine candidates will be individually discussed later.

Trypanosomatid CALPs, similar to mammalian CALPs-calcium-dependent CPs, are involved in a variety of cellular events, e.g., signal transduction, cytoskeleton remodeling, and environmentally-regulated processes<sup>[5]</sup>. On molecular basis, genes encoding CALPs in pathogenic trypanosomatids were categorized into five groups. Members of group 3 are termed small kinetoplastid CALP-related proteins (SKCRPs) that contain only the exclusive N-terminus (the proteolytic domain). Ten, nine, and six SKCRPs were identified in *L. major*, *T. cruzi*, and *T. brucei*, respectively<sup>[6]</sup>. On the other hand, Brazilian reviewers claimed that comparative studies between drug resistant and sensitive isolates of pathogenic

trypanosomatids showed up-regulation of virulence-related genes in infective parasite stages. As genes encoding CALPs were upregulated, the reviewers recommended complete genome sequences of pathogenic trypanosomatids to identify potential chemotherapeutic agents<sup>[7]</sup>.

Programmed cell death (PCD) machinery pathway includes apoptosis and autophagy. The latter is involved in cell turnover through removal of damaged cells and homeostasis regulation for cellular crucial processes; growth and differentiation. Identification of autophagy-related genes (ATGs) showed participation of several proteins in PCD pathway<sup>[8]</sup>. In contrast to mammalian caspases, MCAs were shown to contribute in autophagy, not apoptosis<sup>[9]</sup>. Two other differences were observed; MCAs cleave their substrates after arginine and lysine residues in the P1 position, instead of asparagine in caspases. Second is their activation by calcium<sup>[10]</sup>. Recently, Menna-Barreto<sup>[11]</sup> reviewed trypanosomatid PCD machinery pathway and presented a figure for the distribution of apoptotic or autophagic molecules in *Leishmania* spp., *T. cruzi* and *T. brucei*. Proteins associated with apoptosis (MCAs) were mainly demonstrated in trypanosomes, followed by *L. donovani* and *L. infantum*, and *vice versa* for proteins contributed in autophagy (ATGs) that were mainly assessed in *L. major*, followed by trypanosomes<sup>[11]</sup>.

When the crystal structure of C11 peptidase was characterized, British investigators succeeded to identify an important gene encoding protein termed as Puf nine target 1 (PNT1) in trypanosomatids. The investigators observed that PNT1 showed potential proteolytic activity similar to those of CPs, and was immunolocalized to the kinetoplast DNA. Demonstrated by gene knockout studies, PNT1 proteolytic activity was required for kinetoplast maintenance. Phylogenetic analysis revealed that PNT1 was structurally divergent from those identified in coccidian protozoa<sup>[12]</sup>.

Chagasin, the endogenous inhibitor of CPs (CYS), was described in *T. cruzi*<sup>[13]</sup>, and later in *L. mexicana*, *L. major* and *T. brucei*<sup>[14]</sup>. Functional homology between the identified chagasin suggested a common evolutionary origin. Several evidences were observed suggesting that inhibition of host CPs was the essential role of chagasin<sup>[14]</sup>. Later, an American study identified the crystal structure of chagasin in a complex with its CP. They found that it had unique variant of an immunoglobulin fold with homology to human CD8 $\alpha$ . Chagasin possesses three binding loops (BC, DE, and FG) to fit in the active site groove of human CATHs obstructing substrate binding. Based on the obtained results, the investigators attributed differences in their functional requirements to the highly homologous binding loops evolved in chagasin to be used as scaffold for protein binding<sup>[15]</sup>. Due to lack of a significant identity with CYS classes, it is assigned in a recent classification as clan IX, family I42<sup>[16]</sup>.

#### ***Leishmania* spp.**

Silva-Almeida *et al.*<sup>[17]</sup> attributed variable clinical presentations in leishmaniasis to the high diversity of proteinases, as *L. braziliensis* alone has 97 metallo-, 44 cysteine, and 23 serine proteinases. The reviewers claimed that beside parasite survival and virulence, CPs influenced host immune response *via*: 1) expression of Th1-associated cytokines with production of ILs 4, 5 and 10, as well as IFN- $\gamma$  with inhibition of IL-12 production; 2) association with cleavage of the major histocompatibility complex (MHC) class II gene products; 3) stimulation of CD8<sup>+</sup> T-lymphocytes; and 4) contribution in parasite resistance against killing by macrophages through immunomodulation<sup>[17]</sup>. Later, the same investigators utilized genomic analysis of four *Leishmania* spp. and observed great diversity of genes encoding proteinases. The investigators observed that cysteine and serine proteases, with close percentages, predominate in all analyzed species except *L. braziliensis*, where metalloproteases were the predominant. The investigators concluded that the observed great diversity in *Leishmania* spp. are related to establishment of the infection in their hosts; either the vector gut or the mammalian immune system<sup>[18]</sup>.

In order to survive host antimicrobial activities, intracellular amastigotes have to develop several highly successful strategies including antigen presentation,

nitric oxide (NO), oxygen radical generation and cytokine production. Host immunoevasion is necessary for amastigotes to divide within infected macrophages, and propagate the disease within the host. The molecular mechanisms were discussed and the reviewers concluded that it is certain that significant differences would exist in the mechanisms of host cell manipulation, and this was attributed to the diversity of *Leishmania* spp.<sup>[19]</sup>.

#### **Cysteine proteinases (CPs)**

**Cathepsins:** *Leishmania* CATHs include two CATHs L-like (CPA and CPB) and one CATH B-like (CPC). Other terms for CPA and CPB were used in some publications as *cys1* and *cys2*, respectively. British scientists characterized genes encoding CPB in *L. mexicana*. Its expression level was developmentally regulated, being high in amastigotes, low in metacyclic forms and undetectable in promastigotes<sup>[20]</sup>. A single copy of each gene encoding CPA and CPC was identified<sup>[21]</sup>, and the multi-copy CPB gene of *L. mexicana* was located in a single locus of 19 copies arranged in a tandem repeat<sup>[22]</sup>. Only 5 and 8 copies were detected in *L. chagasi*<sup>[23]</sup>, and *L. major*<sup>[24]</sup>, respectively.

Using gene sequencing and analysis of *cpb* multi-copy gene of *L. mexicana*, three different nucleotide sequences of their repeat units were observed. The copy (*cpb2*), expressed in metacyclic forms, contains four insertions with a 3'-untranslated region (3-UTR). The last 16 copies (*cpb3-cpb18*), expressed in amastigotes, possessed a CTE extension. A copy in between *cpb2* and *cpb3*, termed *cpb2.8*, possessed the CTE and 3-UTR. Expression in metacyclic forms occurred with *cpb2* or *cpb2.8* with its 3-UTR. In amastigotes, expression occurred with *cpb3-cpb18* or *cpb2* with a CTE of *cpb2.8*<sup>[22]</sup>. Later, gene sequencing of 2 *cpb* copies: *cpbE* and *cpbF* revealed that the first was specific to *L. infantum*, while the latter to *L. donovani*, and both copies were different in length and sequence. Phylogenetic studies were conducted in France to compare both copies with those reported in species of *L. mexicana* and *L. donovani* complexes. Results pointed to three issues: 1) both copies shared a sequence pattern specific to *L. donovani* complex; 2) different levels of polymorphism between *L. infantum* and *L. donovani*; and 3) *L. infantum* dermatropic strains showed more polymorphism than viscerotropic strains<sup>[25]</sup>.

It was reported that CATH B-like and L-like CPs in *Leishmania* spp. differ in their structures with different proteolytic mechanisms. When the substrate binds with CATH L-like, it spans the entire channel between the two CP domains, leading to proteolytic activity. Cathepsin B-like has an additional loop that restricts substrate binding under low pH, where at high pH, the loop allows for proteolytic activity<sup>[26]</sup>. Recently, surface plasmon resonance assay was used to track the fate of the CTE of *L. amazonensis* CPB; whether it remained as CTE or was expressed as CPB prodomain in cultured

promastigotes and amastigotes. Using CTE antibodies and a broad spectrum CPI (E-64), results revealed that both stages extracellularly released CTE, while only promastigotes released CTE as CPB prodomain<sup>[27]</sup>.

### **Leishmania CATHs and clinical presentations**

Variable clinical presentations in leishmaniasis were attributed to differences in CPs expression levels and specificities. While *L. mexicana* usually causes chronic lesions, *L. major* often causes self-limited lesions. When *L. mexicana* CPB was transfected into mutant *L. major*, diminished Th1 response and elevated Th2 response with high IFN- $\gamma$  production was observed. Hence, it was concluded that gene expression levels or its substrate specificity are different between both species<sup>[28]</sup>. Sequencing of the gene encoding *L. amazonensis* CPA (*Lmacys1*) showed high similarity with CPB of *L. chagasi* (*Ldccys2*)<sup>[29]</sup>. Similar results were obtained when Canadian investigators observed significant sequence identity in *L. chagasi* CPB with *L. mexicana* CPA, but with lower sequence identity with *L. mexicana* CPB. Accordingly, the differences in gene sequencing and CPs expression between *L. mexicana* and *L. donovani* complexes were confirmed<sup>[30]</sup>.

Several differences within species of *L. mexicana* complex were also observed in megasomes. The latter are large lysosome-like structures in amastigotes linked to CPs expressions. Brazilian investigators immunolocalized CPs in amastigotes of three *Leishmania* spp. They observed variations in megasome size and number either in cultured amastigotes or those obtained from infected animals. Higher volume of megasomes was observed in lesion-derived amastigotes compared with cultured amastigotes in *L. amazonensis* and *L. mexicana*, with the latter 2-3 times higher. In contrast, cultured *L. pifanoi* amastigotes showed a small megasomes volume with low CPs activity and inability to cause lesions in animal models. Accordingly, significant differences of structural organization of CPs distribution within the *L. mexicana* complex was concluded<sup>[31]</sup>. Similar conclusion was obtained when a study conducted in Peru observed 70% sequence identity between *L. braziliensis* and *L. mexicana* CPBs, but with a significant difference in substrate preference. A CPI with proline at P1 showed high affinity to *L. braziliensis* CPB, while *L. mexicana* CPB preferred substrates with phenylalanine, leucine, and asparagine at P1. Accordingly, the investigators suggested amino acid modifications outside the core of the active site cleft for substrate preference<sup>[32]</sup>.

### **Leishmania CATHs for survival and virulence**

A comparative study of proteinases in virulent and avirulent *L. amazonensis* promastigotes showed significant expression of CP activities in virulent promastigotes<sup>[33]</sup>. A postulated mechanism for the role of *Leishmania* CPs to enhance parasite survival in infected macrophages was hypothesized. Significant increased level of activated tumor growth factor- $\beta$

(TGF- $\beta$ ) in *L. chagasi*-infected mice was observed, and was inhibited by addition of a specific CPB inhibitor<sup>[34]</sup>. Brazilian investigators observed that CPs had a key role in growth, viability, and pathogenicity of *L. tropica*, with close similarity to *L. major* rather than *L. mexicana*. However, pathogenicity of *L. tropica* amastigotes in mice was reduced by CPI treatment with similar rate as observed in both *L. major* and *L. mexicana*<sup>[35]</sup>.

Importance of CPB as a major virulence factor for *L. mexicana* complex species was investigated. It was documented that the multiple copies of *cpb* gene express CPB with complementary functions for the sake of survival and virulence of promastigotes and intracellular amastigotes<sup>[36-38]</sup>. Later, Brazilian investigators analyzed profiles of the proteolytic activities of cellular and extracellular CPB and the *Leishmania* metalloprotease virulence factor (gp63). They investigated both virulence factors in isolated virulent and a cultured avirulent *L. braziliensis* strains. Expression of both proteases was exclusively detected in the virulent strain, and was sharply decreased after several *in vitro* subcultures. Growth arrest and virulence decrease of the virulent strain were observed on E-64 addition<sup>[39]</sup>. Similar results were obtained when another group of Brazilian investigators verified that CPB expression was modified during *in vitro* subcultures of *L. braziliensis* promastigotes<sup>[40]</sup>. In 2013, proteomic analysis of *L. donovani* CPB mutant and wild type (WT) identified several modulated proteins. Because the majority of them were lysosomal secreted proteins; the investigators concluded an essential virulence role of CPB through their effect on *Leishmania* secreted proteins<sup>[41]</sup>. Capability of CPB to modulate levels of gp63 in *Leishmania* species was shown. Minimal expression of gp63 in CPB-deficient parasites was sufficient to restore virulence to WT levels<sup>[42]</sup>. On the other hand, the role of the CTE in *Leishmania* virulence was also investigated. The role of *L. pifanoi* CPB-CTE (*Lpcys2* CTE) in early infection was demonstrated. Beside its lysosomal localization, *Lpcys2* CTE was shifted towards the flagellar pocket of cultured amastigotes during early stage of macrophage infection<sup>[43]</sup>.

There is evidence that CPC is also considered an important virulence factor in *Leishmania* spp. due to its partial contribution in increasing *L. chagasi* capacity to induce TGF- $\beta$  expression in human cell cultures<sup>[44]</sup>. Also, assays based on gene suppression showed that *L. infantum* CPA acted as a virulence factor. Mutant clones significantly reduced virulence *in vitro* and *in vivo*. However, re-expression of one *LiCPA* allele was sufficient to retrain virulence in human macrophages *in vitro*, but not *in vivo*. The investigators confirmed that CPA had an essential role for the host-parasite interaction, and not for promastigotes replication<sup>[45]</sup>. In contrast, another study conducted in Ethiopia observed that expression of CPA and CPB in *L. aethiops* was during the stationary phase of development<sup>[46]</sup>.

**Leishmania CATHs and host immune response**

One of the factors influencing outcome of *Leishmania* infection is the balance between Th1 response antagonizing infection, and Th2 response in the favor of parasite survival. *Leishmania* CPB have an essential role in the differentiation of functional CD4<sup>+</sup> T cells. Using a specific CATH B inhibitor, Maekawa *et al.*<sup>[47]</sup> observed modulation of host immune response from Th2 to Th1 type in susceptible BALB/c mice infected with *L. major*. Also, it was suggested that *Leishmania* CPB and mammalian CATH L had the same function as antigen-processing proteases, but in a different manner. While *Leishmania* CPB modulated host immune response by Th2 upregulation and/or Th1 suppression, the latter processed *Leishmania* antigens only to Th1 response<sup>[48]</sup>. Later, the immunogenic properties of *L. infantum* CPA and CPB mature domains were investigated against sera from active and recovered cases of visceral leishmaniasis (VL). Intensive recognition was detected in both sera with higher recognition toward CPB<sup>[49]</sup>.

Several mechanisms were postulated for CPs modulation of host immune response. In mice infected with mutant *L. mexicana* (deleted *cpb* multi-copy gene), several hypotheses were postulated for suppression of Th1 immune response; either indirectly or directly. The indirect mechanism involved inhibition of antigen presentation cells *via* CP-mediated degradation of MHC class II molecules and inhibition of T cell proliferation. Direct mechanisms involved altering Th1 pathways or their crucial transcription factors. Accordingly, the investigators suggested utilizing CPB specific inhibitors for treatment of chronic leishmaniasis<sup>[28]</sup>. Similar results were obtained when the investigators inserted only *cpb2.8* or *cpb2* gene into *cpb*-mutant *L. mexicana*. Results revealed failure to restore either Th2 response or virulence. Re-expression of *cpb* gene significantly increased lesion growth accompanied by Th2 response with increased production of IL-4, IgG1 and IgE<sup>[38]</sup>. In *L. mexicana*, two other mechanisms were postulated for CPB down regulation of protective Th1 immune response; IFN- $\gamma$  production and inhibition of IL-12 production by infected macrophages<sup>[50]</sup>. Another reported mechanism of *Leishmania* CPs was the proteolytic activity of kininogens liberating kinins. The latter were recognized as signals alerting the innate immune system. The investigators demonstrated that both *L. donovani* and *L. chagasi* activated kinin pathway to enhance microvascular responses; i.e. modulation of innate immunity in VL. Interestingly, kinin pathway was inhibited using irreversible CPIs<sup>[51]</sup>. Later, *L. mexicana* CPB was reported to have a crucial role in alteration of macrophage signaling pathways and inactivation of transcription factors leading to nuclear failure to respond to IFN- $\gamma$  stimulation<sup>[52]</sup>.

As CTE-CPB was reported to influence the interaction of parasites with the host immune system, the investigators hypothesized that the CTE is hydrolyzed when CPB is processed to its mature form

and was secreted into the extracellular environment<sup>[23]</sup>, and inside host cells<sup>[53]</sup> altering host immune system. In murine leishmaniasis models, BALB/c mice are highly susceptible, while CBA mice are mildly resistant, and this was attributed to inefficient Th1 response rather than a dominant Th2 response<sup>[54]</sup>. The protective potentiality of the CTE of *L. infantum* CPA was investigated in BALB/c mice. The investigators observed increased levels of IgG2a and IL-5, and concluded that recombinant CTE-CPA was immunogenic and displayed both Th1 and Th2 immune responses in experimental VL<sup>[55]</sup>.

To elucidate the molecular mechanism of CTE in Th1/Th2 immunomodulation for the favor of parasite survival, three studies were conducted in two mice models. In the first study, the investigators conducted *in silico* mapping to predict three peptides (epitopes) in the CTE of *L. amazonensis* CPB that were suggested to bind with MHC molecules. Results revealed more intense Th1 response only in infected CBA mice, but a Th2 response was observed in both models<sup>[56]</sup>. This suggested that inefficient Th1 response contributed to increased susceptibility of BALB/c mice. In the second study, the investigators demonstrated diversity of stimuli by the selected peptides on Th1/Th2 response for the best favor of parasite survival. *In silico* simulation studies showed that there were essential amino acid residues in one of them (PI) to help binding with the MHC cleft. Molecular docking studies demonstrated confirmatory evidence for the relation between PI capacity and expression of specific cytokines for Th1/Th2 balance. The distinct immunomodulatory effects on cytokines (Th1/Th2 balance) was attributed to the interaction between MHC classes and PI in the CTE<sup>[57]</sup>. Analyzing gene expression of *L. amazonensis* CPB and CPC as well as host gene expression related to T cell response balance (Th1/Th2), namely MHC classes I and II was conducted in the third study. Expression of CPB predominated over that of CBC in both mice models, while MHC classes I and II had higher expression levels in CBA and BALB/c mice, respectively<sup>[58]</sup>.

**Leishmania CATHs and autophagy**

*Leishmania* CPA and CPB proved to be essential for autophagy, an essential process during differentiation and transformation (promastigotes $\leftrightarrow$ amastigotes). Autophagosomes were much more demonstrated during promastigotes conversion to amastigotes than the other transformation form. Deletion of both genes inhibited autophagy pathway, and prevented metacyclogenesis both *in vitro* (macrophages) and *in vivo* (mice)<sup>[59]</sup>. In addition, an essential role played by CPC was reported in *Leishmania* PCD machinery. As previously reported, the strategy "protozoa commit suicide to survive" was suggested<sup>[60]</sup>. That is to say "dead promastigotes enable the intracellular survival of the viable amastigotes influencing parasite virulence". In their study, El-Fadili *et al.*<sup>[61]</sup> identified *Leishmania* CPC as an efficient substrate of a pan-caspase inhibitor. The investigators provided evidence for induction of PCD by

various stress conditions. The induced high expression of *Leishmania* CPC activates PCD machinery pathways controlling the density of *Leishmania* population in its host<sup>[61]</sup>.

### ***Leishmania* CATHs as diagnostic markers**

A group of Belgium scientists utilized *Leishmania* CATHs for the genetic characterization of *Leishmania* species. Application of restriction fragment-length polymorphism (RFLP) of PCR-amplified sequences of the multi-copy gene encoding CPB, characterized 15 references strains of *L. donovani* complex. The investigators validated their protocol because PCR-RFLP allowed direct characterization of *Leishmania* species in human tissue samples<sup>[62]</sup>. Other Belgium investigators developed a rapid PCR technique to differentiate between five *Leishmania* spp. and it was validated in cultured isolates<sup>[63]</sup>. One year later, a third Belgium publication reported use of three nuclear loci encoding three markers; g63, CPB, and hydrophilic acylated surface protein B markers to genotype thirty-four *L. donovani* clinical isolates obtained from Nepal. Results showed eight genotypes using combined markers. The investigators recommended further studies to utilize these markers to study the epidemiological factors involved in parasite populations, distinguish relapses from re-infection, and monitor strains spread<sup>[64]</sup>. Gene encoding CPB was also investigated to design primers for routine diagnosis of leishmaniasis caused by *L. aethiopica*. The investigators claimed that these primers were able to differentiate between *L. aethiopica* from other *Leishmania* spp.<sup>[65]</sup>.

Tunisian investigators published two articles in 2013 utilizing *cpb* gene. The first reported a RFLP-PCR with selected primers to differentiate between several Tunisian *Leishmania* isolates. Utilizing phylogenetic analysis, the primers enabled the investigators to identify a new Tunisian species, *L. killicki* which was an evolved isolate from the *L. donovani* complex originated from East Africa<sup>[66]</sup>. In the second report, they used loop mediated isothermal amplification (LAMP) to amplify six primers targeting *L. infantum cpb* gene. No cross reaction was observed with other *Leishmania* spp. and *T. cruzi* or human genomic DNA. Although moderate sensitivity and high specificity were observed, LAMP showed the highest *kappa* value (0.34 fair agreement). Moreover, relative stability of LAMP reagents suited its use in field blood samples<sup>[67]</sup>.

Three publications were issued by a group of Brazilian scientists<sup>[68-70]</sup> investigating recombinant *L. chagasi* CPA (rLdcccys1) as a serodiagnostic marker in VL caused by *L. chagasi* in Latin America. It was utilized to assess cellular immune responses in naturally VL-infected humans and dogs. The investigators detected elevated levels of IFN- $\gamma$  only in asymptomatic subjects, or associated with ILs 4 and 10 in symptomatic subjects<sup>[68]</sup>. Also, it showed high sensitivity rates for detection of specific antibodies in sera obtained

from patients with active VL. Results revealed 98% and 99% sensitivity to lysates of promastigote and amastigote, respectively, with no cross reactions with sera of Chagas's disease patients<sup>[69]</sup>. Later, it was used to diagnose canine VL and to discriminate between clinical and subclinical infections. Results revealed no cross-reactivity with sera obtained from patients with Chagas' disease, however little reactivity was detected with sera from dogs with babesiosis. Delayed type hypersensitivity (intradermal response) was also determined, and the results revealed that all asymptomatic dogs showed positive results with a peak at 48 h. In contrast, no significant response was observed in the symptomatic dogs<sup>[70]</sup>.

### ***Leishmania* CATHs as vaccine candidates**

*L. chagasi* CPA (Ldcccys1) in DNA construct and its recombinant form were utilized to investigate their potentiality to produce protective responses in BALB/c mice challenged with *L. chagasi* amastigotes. Mice immunized by both protocols together induced significant secretion of IFN- $\gamma$ , NO and IgG2a antibodies suggesting a predominant Th1 response<sup>[71]</sup>.

Due to its significant induction of Th2 immune response, British investigators suggested using *L. mexicana cpb2.8* gene as a potential vaccine candidate if administered with a suitable adjuvant<sup>[72]</sup>. Colombian investigators also succeeded to achieve significant protection against *L. mexicana* challenge infection utilizing *cpb*-mutant attenuated live promastigotes. Significant lower levels of Th2-associated cytokines (IL-10 and TGF- $\beta$ ) were observed in comparison to WT infection<sup>[73]</sup>. Later, six *Leishmania* antigens were evaluated as vaccine candidates for VL. To measure vaccine validity, the investigators found that out of the six tested cytokines, only IFN- $\gamma$  differentiated cured VL patients from non-exposed individuals. The most immunogenic and protective antigen was CPB followed by sterol 24-c-methyltransferase. The investigators recommended further studies for clinical development of a valid vaccine against VL<sup>[74]</sup>.

Utilizing both CPA and CPB as a vaccine candidate was reported in several publications. Iranian investigators utilized recombinant CPA and CPB to control VL in dogs. Results revealed that almost all vaccinated dogs showed strong delayed type hypersensitivity responses compared to control dogs<sup>[75]</sup>. Two delivery systems (electroporation and nanotechnology) of a vaccine composed of *L. donovani* A2 antigen with *L. infantum* CPA and CPB without its CTE (CPB2.8 $\Delta$ CTE) were evaluated. Both delivery systems induced protective immunity associated with a strong Th1 immune response, as well as high levels of IgG antibodies and NO production. Histopathological studies and parasite burden showed similar protection levels<sup>[76]</sup>. Nanotechnology was also utilized in another study to develop a protective vaccine against CL. Recombinant *L. major* CPA and CPB were conjugated

with PLGA nanoparticles to enhance immune response in BALB/c mice. After *L. major* challenge infection, the investigators concluded that vaccination using CPs combined with nanoparticles produced the most significant protection<sup>[77]</sup>. Recently, outbred dogs were vaccinated with nonpathogenic *L. tarentolae* combined with *L. donovani* A2 antigen, CPA and CPB2.8ΔCTE against *L. infantum* challenge infection. Vaccinated dogs showed strong delayed type hypersensitivity associated with low bone marrow parasite burden. After challenge infection, highest percentage of subclinical infection and the lowest percentage of symptomatic stage were observed. Because dog is the major reservoir host for VL, vaccination against canine leishmaniasis was recommended as a strategy to control VL<sup>[78]</sup>.

Applying *L. infantum* CPC as a vaccine candidate was also investigated. Significant high ratio of IgG2a/IgG1 associated with NO and IFN- $\gamma$  production, were observed in the vaccinated mice compared to the control group<sup>[79]</sup>. Immuno-informatics was utilized to predict three specific immunogenic peptides in *L. donovani* CPC. The predicted peptides (P 1-3) showed low sequence similarity with *Homo sapiens*, suggesting high potentiality and specificity to elicit human T-cell immune response. Interestingly, these predicted peptides were highly conserved in *L. major*, *L. infantum* and *L. chagasi*. The investigators selected two peptides (P1 and P3) because they showed strong binding affinity with the HLA A02 allele *in vitro*. Significant potentiality of individual P1 or P3 to generate protective immune response (CD8<sup>+</sup> T-cell proliferation) was observed in treated VL patients compared to healthy controls. Development of DNA vaccine against VL employing combined P1 and P3 was recommended<sup>[80]</sup>.

### Calpains (CALPs)

Employing whole-genome analysis, a total of 27 CALP-like proteins were characterized in *L. major*<sup>[6]</sup>. Using anti-CALP antibodies, CALP-like molecules on the cell surface of *L. amazonensis* were localized. The investigators observed growth arrest on addition of a specific CALP inhibitor<sup>[81]</sup>. Caldonopain, a CALP-like proteinase, was identified in *L. donovani* cytosolic fraction. Because it showed a calcium-dependent proteolytic activity, caldonopain was suggested as novel drug target against VL<sup>[82]</sup>.

Utilizing gene expression microarray technology, the investigators observed a double-fold higher expression of five proteins in *L. donovani* isolated from patients with post kala-azar dermal leishmaniasis in comparison with those isolated from VL patients. Among them, a short protein with significant homology to trypanosomatids CALPs, was identified. The investigators suggested that *L. donovani* CALP might contribute to drug resistance in VL<sup>[83]</sup>. Similarly, a proteomic analysis between drug-resistant and sensitive *L. donovani* strains isolated from patients with VL was conducted. In the resistant strain, down

regulation of the gene encoding small kinetoplastid calpain-related protein (SKCRP14.1) was observed. It was concluded that *L. donovani* CALP increases the parasite susceptibility<sup>[84]</sup>. To identify biomarkers for miltefosine resistance in VL patients, a recent study used cultured splenic aspirates from two groups of VL patients; one successfully treated with miltefosine and another that had relapsed after initial cure. The investigators only identified a CALP-like protein with significant over expression in relapsed patients. It was concluded that *Leishmania* CALP can be used as a potential biomarker of miltefosine resistance<sup>[85]</sup>.

### Metacaspases (MCAs)

In leishmaniasis, reactive oxygen species, including H<sub>2</sub>O<sub>2</sub>, are produced by anti-leishmanial drugs or infected macrophages to participate in killing intracellular *Leishmania*. A group of researchers found that pre-treatment of *L. donovani* promastigotes with a specific CPI reduced the number of apoptotic cells, and prevented DNA fragmentation. Role of *Leishmania* MCA (clan CD, family C14) in activation of caspase-like proteins leading to PCD was suggested<sup>[86]</sup>. Similar results were obtained using E-64, suggesting involvement of *Leishmania* MCA in PCD, similar to intracellular protozoa<sup>[87]</sup>.

In 2007, a single *mca* gene was identified in *L. major*. The investigators observed that the proteolytic activity of *LmMCA* was auto-processed, and it preferred substrates with arginine in the P1 position, but not caspase substrates<sup>[88]</sup>. To elucidate MCA molecular role involved in PCD, genomic sequence analysis revealed three main domains; N-terminus; catalytic domain and proline rich C-terminus<sup>[89]</sup>. To predict the relevant cleavage sites for auto-processing of *LmMCA* catalytic domain, Swiss investigators suggested R63, R315 at the N-terminus, and R298 at the C-terminus domains<sup>[90]</sup>. From the previous studies, it was concluded that biochemical characterization and identification of *LmMCA* natural substrates<sup>[89]</sup> and/or identification of relevant cleavage sites of its auto-processing<sup>[90]</sup> would help in development of novel drugs against leishmaniasis.

In contrast to *L. major* and similar to *L. infantum*, two genes encoding MCAs were cloned and characterized in *L. donovani* (*LdMCA1* and *LdMCA2*). Their sequence showed 98% homology and possessed a characteristic C-terminus proline-rich domain. The investigators observed mRNA transcripts in promastigotes and axenic amastigotes, with significant more *LdMCA1* transcripts in axenic amastigotes. Pretreatment with H<sub>2</sub>O<sub>2</sub> induced *LdMCA*s overexpression<sup>[91]</sup>. Also, another study revealed MCAs over-expression of cultured *L. infantum* promastigotes 6 h after treatment with miltefosine. The investigators suggested that MCAs initiated signaling for apoptosis pathway<sup>[92]</sup>. The roles of *LdMCA1* and heat shock protein 70 (HSP70) in PCD of *L. donovani* were identified in gene knockdown

studies. Individual gene knockdown showed marked cell death, while simultaneous knockdown led to maximum damage. Both *L. donovani* MC1 and HSP70 were suggested as drug targets<sup>[93]</sup>. For *L. mexicana*, British investigators generated null mutant clones of *L. mexicana* promastigotes and amastigotes (deletion of *mca* gene), and they observed their differentiation *in vitro* and virulence *in vivo*, respectively. It was concluded that *LmMCA* was only an essential survival factor for amastigotes replication inside the mammalian host<sup>[94]</sup>.

#### Other understudied CPs

*Leishmania* spp. possess two ATG4 CPs (ATG4.1 and ATG4.2) for their autophagy process that is essential for cell differentiation. However, it was demonstrated that ATG4.2 expression, not ATG4.1, increased parasite virulence through its essential role in autophagy<sup>[95]</sup>. Otubain (clan CA, family C65), a deubiquitinating enzyme (DUB) with an essential role in regulating several eukaryotic processes, was recently characterized in *L. infantum* (*OtuLi*)<sup>[96]</sup>. It is worth mentioning that ubiquitination is a process in which an ubiquitin molecule (Ub) covalently binds to a substrate to regulate a specific signaling pathway<sup>[97]</sup>. Localization of *L. infantum* otubain cytoplasm in promastigotes indicated its essential role in pro-inflammatory response of stimulated murine macrophages<sup>[96]</sup>.

#### Cysteine proteinase inhibitors (CPIs)

Several studies were conducted investigating peptide proteinase inhibitors in treatment of leishmaniasis. A CATH B inhibitor, RT-01 (a synthetic organotellurane) inhibited the growth of cultured *L. amazonensis* amastigotes and promastigotes. Significant delay in cutaneous lesions development and decreased parasite intensity were observed after intra-lesional administration for one month<sup>[39]</sup>. Also for treatment of CL, subcutaneous injections of DPPE 1.2, a metalloproteinase inhibitor, showed significant inhibitory activity against *L. amazonensis* CPB. Its application in experimentally infected mice for one month significantly decreased lesion size and produced 97% reduction of parasite burden<sup>[98]</sup>. In another study, one of the organotelluranes, RF07, was investigated for treatment of VL. Intraperitoneal injection for one month produced almost complete reduction in the parasite burden<sup>[99]</sup>. Recently, derivatives of dipeptidyl nitriles, selective inhibitors of human dipeptidyl aminopeptidase 1 (DPAP1), were investigated. Significant growth inhibition of *in vitro* cultured promastigotes of *L. amazonensis* and *L. infantum* was observed. Unfortunately, DPAP inhibitors caused cytotoxic effects on infected macrophages that impaired further analysis of the amastigote infection<sup>[100]</sup>.

Due to the significant potentiality of vinyl sulfones as leishmanial CPIs, several non-peptide and peptidomimetic inhibitors were investigated. Three studies were conducted by a group of scientists from Germany and Venezuela utilizing synthetic

aziridine-2,3-dicarboxylates. It was demonstrated that compounds 13b and 13e impaired *in vitro* promastigote growth, and decreased macrophages infection rate. However, when compared to amphotericin B *in vivo*, both compounds exhibited less potency suggesting their direct inhibitory activity against *Leishmania* CPB<sup>[101]</sup>. Apoptotic markers were observed when both compounds were investigated targeting *Leishmania* CPC. Unfortunately, they caused inhibition of host CATH L<sup>[102]</sup>. To discriminate between host and parasite CPs, more selective inhibitors were synthesized based on both compounds. Compound s9 showed selective inhibitory potency against *L. major* CPC and *L. mexicana* CPB2.8, but not against mammalian CATHs B and L. It showed excellent IC<sub>50</sub> against *L. major* promastigotes and amastigotes *in vitro*<sup>[103]</sup>.

Compounds containing an azide moiety were investigated for their inhibitory potency against CPB2.8ΔCTE. They were also tested for cytotoxicity against murine macrophages *in vitro*, and mutagenicity towards prokaryotic and eukaryotic cells. An alkyl-phosphocholine derivative (No. 4) exhibited the most potency against *Leishmania* promastigote and amastigotes, compared to pentamidine and amphotericin B, with lower toxicity as well as mutagenicity<sup>[104]</sup>. Recently, a new selective aziridine-based inhibitor was designed and it showed a time-dependent inhibition of CPB2.8ΔCTE. Docking studies confirmed the strong ionic interaction with the active site<sup>[105]</sup>.

Moreover, nine benzophenones were evaluated for their potential inhibitory activity against *L. amazonensis* CPB2.8 and CPB3 as well as cruzain (CRZ), the major *T. cruzi* CP. Only one derivative (1c) exhibited selective activity towards the investigated CPs. When compared to amphotericin B, significant *in vitro* killing of intracellular amastigotes was observed<sup>[106]</sup>. Utilizing enzymatic screening, thiophenes showed a moderate to excellent inhibitory activity by binding to CPB2.8ΔCTE active site. The most promising compounds were selected for further evaluation regarding *in vitro* assays, docking studies as well as cytotoxicity and mutagenicity. Almost all investigated compounds, except one, acted as irreversible covalent inhibitors. Only compound 2b showed a reversible covalent mechanism and was identified as a novel drug against leishmaniasis<sup>[107]</sup>. Similarly, searching in library database for compounds possessing a benzimidazole or an indole ring as scaffold, twelve compounds were synthesized for further evaluation. Four of them (9a-d) showed selective inhibitory activity against *rLmCPB2.8ΔCTE*, without targeting mammalian CATHs. However, no clear correlation was observed between inhibitory activities against *rLmCPB2.8ΔCTE* and those against intracellular amastigotes. Although the most active compound (9d) proved to be more cytotoxic, it was the least permeating blood brain barrier, i.e. no neurotoxicity, when compared with 9a-c. It was



concluded that 9d might be a new lead CPI for further drug design for treatment of leishmaniasis<sup>[108]</sup>.

As homology modeling of the mature *L. mexicana* CPB2.8 showed significant difference from bovine CATH B, the possibility of its evaluation as a good drug target was raised. A high throughput screening (HTS) for inhibitory compounds against rLmCPB2.8ΔCTE and bovine CATH B was conducted. Docking experiments with structural activity relation (SAR) analysis revealed that compounds containing triazine nitriles, semicarbazones and thiocarbazones suited better as peptidomimetic inhibitors of CPB2.8ΔCTE rather than bovine CATH B<sup>[109]</sup>. Using a computer-aided approach, followed by homology modeling and docking studies, synthesized chalcone and chalcone-like compounds were evaluated for their inhibitory potency against *Leishmania* CPB. The promising compounds were selected for further *in vitro* evaluation as anti-leishmanial drugs as well as their cytotoxicity towards *Vero* cells. Only two compounds exhibited anti-leishmanial efficacy comparable to amphotericin B, with low cytotoxicity<sup>[110]</sup>.

### Cystatins (CYSs)

British investigators observed that *Leishmania* promastigotes null mutants in the gene encoding chagasin were similar to WT parasites. Host-parasite interaction *via* modulating host CPs activity was suggested as a main role for *Leishmania* chagasin<sup>[111]</sup>. As previously described for chagasin structure, the investigators claimed that it was the only endogenous CPI with a cadherin-like immunoglobulin domain to be discovered in a non-metazoan. They also demonstrated the essential role played by the mobile loop in host-parasite interaction<sup>[112]</sup>. To evaluate the influence of chagasin in modulation of host immune response, its potentiality as novel drug target and/or vaccine candidate was studied. Ability of experimentally infected mice to control challenge infection with WT parasites was observed when vaccinated with chagasin over-expressing clone<sup>[113]</sup>. However, recombinant chagasin of *L. mexicana* did not inhibit midgut proteolytic enzymes extracted from *Lutzomyia longipalpis*, i.e., had no role in protection of *Leishmania* parasites in the vector<sup>[114]</sup>.

### *Trypanosoma* spp.

#### American trypanosomiasis

#### Cysteine proteinases (CPs) of *T. cruzi*

#### Cruzipain (CRZ)

**Historical background:** Thirty years ago, cruzipain (CRZ), was known as a glycoprotein antigen (GP57/51). Its catalytic domain possessed N-terminus homologous to CP sequence. It degraded proteins at a wide pH range and elicited T cell immune response<sup>[115]</sup>. Interestingly, CRZ displayed dual CATHs L and B specificity<sup>[116]</sup>. Several genes encoding CRZ (up to 130) were reported giving rise to CRZ isoforms with varying degrees of similarity<sup>[117,118]</sup>. Its crystal structure consists of a

single polypeptide chain represented by two domains;  $\alpha$ -helical L domain and antiparallel  $\beta$ -sheet R domain<sup>[119]</sup>. It was highly accumulated and active in reservosomes, large round lysosome-like organelles located at the posterior end of *T. cruzi* epimastigotes<sup>[120]</sup>. Expression of CRZ was analyzed among three virulent *T. cruzi* isolates and their attenuated counterparts. Several bands of lower density in all attenuated strains were observed. Analysis of more isolates was recommended to investigate if attenuation was associated with change in CRZ sequence<sup>[121]</sup>.

**Immunolocalization:** It was reported that *T. cruzi* epimastigotes use two endocytic vesicles; cytostome/cytopharynx complex and the flagellar pocket membrane, to ingest extracellular macromolecules to be stored in reservosomes<sup>[122]</sup>. Using a monoclonal antibody (mAb) against recombinant CRZ localized a 50 kDa protein in reservosomes<sup>[123]</sup>. Later, endocytosis was visualized in intracellular amastigotes, and the digested extracellular macromolecules were stored in CRZ-positive lysosome-related structures (LRSs). The investigators suggested that LRSs were functionally related to reservosomes in epimastigotes<sup>[124]</sup>.

**Genetic diversity:** Expression levels and genetic diversity of CRZ isoforms varied among *T. cruzi* isolates as well as among *T. cruzi*-like species. This variation correlated with the observed levels of cellular invasion, differentiation, virulence and pathogenicity in the isolated strains. Eighty sequences of genes encoding CRZ from 25 *T. cruzi* isolates and ten sequences of homologous CRZ encoding genes from *T. cruzi*-like species were comparatively analyzed. It was concluded that CRZ was a diagnostic and biogenetic marker beside its potentiality as drug target or vaccine candidate<sup>[125]</sup>.

**Survival and virulence:** The capability of host cells invasion by cultured trypomastigotes proved to be mediated by CRZ. Its expression activated host kinase II to degrade kinin. This was associated with overexpression of bradykinin B2 receptor. Inhibition of bradykinin degradation by kinase II potentiated host invasion. Accordingly, CRZ was suggested as a virulence factor<sup>[126]</sup>. Later, Scharfstein<sup>[127]</sup> reviewed CRZ interactions with host CPs and endogenous CPI (chagasin). He claimed that CRZ interacted with both extravascular infection sites to produce Chagas' disease dynamics yielding inflammatory reactions associated with host immune response<sup>[127]</sup>.

Fibronectin was reported to be involved in microbe's adherence to host cells, serving as a barrier to prevent parasite invasion and migration. In an *in vitro* study, it was observed that when exogenous fibronectin exceeds CRZ concentration, a reduction occurs in metacyclic invasion into host cells. It was concluded that CRZ proteolytic activity played an essential role in fibronectin degradation, facilitating entry of the metacyclic forms, i.e. host cell invasion<sup>[128]</sup>.



In addition, CRZ activated host TGF- $\beta$  signaling pathway that facilitates host cells invasion by *T. cruzi*. Addition of CATH inhibitor or chagasin inhibited and prevented TGF- $\beta$  activation, respectively. Also, *T. cruzi* invasion and intracellular growth were inhibited on addition of CATH CPI or anti-TGF- $\beta$  antibodies to *Vero* cell cultures. Therefore, CRZ was suggested as drug target in treatment of Chagas' disease<sup>[129]</sup>.

For the sake of parasites survival in Chagas' disease, two studies conducted in Argentine showed that CRZ devoid of enzymatic activity prevented apoptosis of cardiomyocytes. Two signaling pathways induced *in vitro* by CRZ were identified<sup>[130,131]</sup>. Another Argentinian study postulated that IL-6 mediates cell survival and host innate immune response through activation of transcription factor STAT3 *via* the glycoprotein gp130. The investigators observed that CRZ devoid of its enzymatic activity triggered toll-like receptor 2 (TLR2) to produce IL-6 that prevented apoptosis of cardiomyocyte *in vitro*. They also observed that cultured cardiomyocytes treated with active CRZ did not save them from apoptosis in spite of high IL-6 levels. This was attributed to CRZ proteolytic activity to inhibit STAT3 phosphorylation and to cleave recombinant gp130 *in vitro*. Accordingly, the investigators hypothesized that CRZ modified its strategy to confer cardiomyocytes protection induced by IL-6 as anti-apoptotic factor. Utilizing CRZ specific inhibitors was suggested to improve host immune activation with its cardio-protective effects<sup>[132]</sup>. Later, long term culture passages in cell lines of a single *T. cruzi* clone generated two different clones, one high and one low virulent strain. The investigators observed that the low virulent strain was three to five-fold less infective to mouse cardiomyocytes than the other. They also observed high CRZ expression from the highly virulent strain; hence it was concluded that CRZ contributed in *T. cruzi* growth, survival and virulence<sup>[133]</sup>.

Not only limited to vertebrate host, CRZ was also required for survival of *T. cruzi* in its vector. Adherence of *T. cruzi* epimastigotes to *Rhodnius prolixus* posterior midgut cells both *ex vivo* and *in vivo* was linked to CRZ. The investigators observed significant increased surface CRZ expression from epimastigotes isolated after passage in *R. prolixus*. Treatment with CYSS, E-64, chagasin and anti-CRZ antibodies significantly decreased parasites adhesion. The investigators concluded that CRZ was essential for successful colonization of *R. prolixus* by *T. cruzi*<sup>[134]</sup>.

**Elicitation of host immune response:** CRZ expression was responsible for induction of strong humoral and cellular immune response. Host antibodies were bound with cardiac myocin; i.e. autoimmune response, resulting in cardiological manifestations associated with Chagas' disease<sup>[135]</sup>. Host response immunomodulation was also reported because CRZ expression obliged infected macrophages to lose

their pathway for production of pro-inflammatory cytokines. It was shown that CRZ up-regulated Th2 immune response by increased production of IL-10 and TGF- $\beta$ , and down-regulated NO production. Macrophage triggering by CRZ expression, also known as alternative macrophage pathway; was suggested for the favor of parasite survival<sup>[136]</sup>. The role of CRZ in host immunoevasion was elucidated when the investigators evaluated macrophage response pathway in early infection with CRZ-deficient parasites in comparison to WT. In CRZ-deficient parasites, a rapid activation of host macrophage pathway was observed with increased levels of P65, a component of nuclear factor *kappa*-light-chain-enhancer of activated B cells (NF- $\kappa$ B). It is worth mentioning that NF- $\kappa$ B family members regulate innate and adaptive immune responses. In less than one hour, there was inability of intracellular growth and failure to survive inside infected macrophages. The investigators concluded that CRZ degraded NF- $\kappa$ B P65 *via* its proteolytic activity to facilitate *T. cruzi* survival and replication<sup>[137]</sup>.

As it was previously reported that CRZ domain possessed unusual sulfate groups in its N terminus extension, BALB/c mice were immunized with purified CRZ and with its extension before and after desulfation treatment. Results revealed that sulfate groups were essential for IgG2b production and generation of memory T-cell responses. Ultrastructural alterations in heart tissue confirmed the essential role played by the sulfate groups<sup>[138]</sup>. Another team of scientists from Argentina and Germany investigated the role of sulfate groups in host immune response. Ability of glycopeptides containing sulfated groups to immunomodulate host immune response was observed for the sake of parasitemia and *T. cruzi* virulence<sup>[139]</sup>. On the other hand, host innate immune response is mediated by production of reactive oxygen species (ROS) *via* phagocytes. It was demonstrated that CRZ induced ROS production in splenocytes from non-immune and immune mice as well as in macrophage cell lines. It was also observed that CRZ increased production of IL-6 and IL-1 $\beta$ <sup>[140]</sup>.

**Egress:** Role of CRZ in host-cell membrane permeability during egress cascade was described only in an American study. The investigators showed that chronically *T. cruzi*-infected mice produced antibodies that were bound with the surface of infected cells reducing parasite egress<sup>[141]</sup>.

### Applications

**Biogenetic marker:** Apolipoprotein A-I (Apo A-I) was previously identified as a potential diagnostic marker for Chagas' disease. Canadian investigators utilized Western blot to demonstrate Apo A-I susceptibility to CRZ proteolytic activity. The investigators concluded that CRZ contributed in production of Apo A-I truncation in the biomarker set<sup>[142]</sup>. On the other hand, dendrogram analyses based on presence/absence

of CRZ and other proteases bands showed 50-60% similarity between the assigned assemblages of *T. cruzi* (TCI and TCII) field isolates. It was demonstrated that protease expression profile of TCII isolates, in general, presented higher heterogeneity compared to TCI. However, CRZ expression did not show heterogeneous profile in TCII isolates<sup>[143]</sup>. Similar results were obtained when the same group of researchers investigated CRZ heterogeneity in 16 *T. cruzi* field isolates (eight of each assemblage). Stronger CRZ fluorescence labeling was observed more in TCI than in TCII isolates. Significant increased metacyclogenesis levels *in vitro* were also observed in TCI isolates<sup>[144]</sup>.

Utilizing anti-CRZ antibodies to identify CRZ profile in four *T. cruzi* sylvatic Z3 isolates showed an additional protein band with MW 80 kDa only in one strain (SMM10). All strains showed a 40 kDa protein band resembling that of CRZ activity<sup>[145]</sup>. Later, another study revealed molecular markers in sequencing genes encoding CRZ in related *T. cruzi* and *T. cruzi*-like species. Overall, the investigators identified valuable diagnostic markers for phylogenetic analyses of trypanosomes<sup>[125]</sup>.

**Vaccine candidate:** Recombinant CRZ with toll like receptor (TLR2/6), as an adjuvant, produced strong systemic and mucosal antibody responses. Significant cell-mediated immunity, associated with reduction of parasite burden and tissue damage was observed in immunized mice<sup>[146]</sup>. In CRZ immunization, increased survival rate of B cells with increased IL-4 production in BALB/c (susceptible to cardiac autoimmunity) compared to C57BL/6 mice (resistant), was observed. The investigators concluded that CRZ immunization contributed in increasing host autoimmunity<sup>[147]</sup>. The role of attenuated *Salmonella enterica*, as a delivery system, carrying plasmid encoding CRZ was investigated. Naked CRZ-DNA or *Salmonella* CRZ DNA vaccine co-administered with a plasmid encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), as an adjuvant were used. Individual vaccine showed significant protective and sustained Th1 response during acute infections, while combined vaccines prevented tissue damage during chronic infections. The investigators concluded that vaccine immunotherapy alone or in combination with other drugs represented a promising alternative regimen of treatment of Chagas' disease<sup>[148]</sup>.

### Calpains (CALPs)

Employing whole-genome analysis, a total of 24 CALP-like proteins were characterized in *T. cruzi*<sup>[6]</sup>. Expression of the gene encoding TcCALP was identified during metacyclogenesis. Also, expression of CALPs and CALP-related proteins was associated with nutritional or environmental stress conditions. The investigators observed that TcCALP was 2.5 times more abundant in vector epimastigotes under nutritional stress than in epimastigotes growing in culture medium<sup>[149]</sup>. A calpain inhibitor was utilized to identify CALP-like CPs

in *T. cruzi* epimastigotes. Significant *in vitro* growth rate reduction was observed after 48 h. In addition, the investigators observed that TcCALP expression was reduced if epimastigotes were kept in axenic cultures for a long period<sup>[150]</sup>. Moreover, utilizing proteomic analysis of *T. cruzi* isolates with selected *in vivo* and *in vitro* resistance to benzimidazole showed over expression of some proteins in resistant isolates, CALPs were among them. The investigators attributed this over expression to adaptation of the parasite to the unfavorable drug stress conditions<sup>[151]</sup>. Two other studies showed that CALP inhibitor inhibited the growth of *T. cruzi* clinically relevant forms<sup>[152]</sup>, and significantly reduced epimastigotes adherence to the insect luminal midgut<sup>[153]</sup>.

### Metacaspases (MCAs)

Genomic analysis of *T. cruzi* revealed two genes encoding MCA3 and MCA5. While gene encoding MCA3 was composed of 16 copies per haploid genome, arrayed in two tandems, MCA5 was a single copy gene<sup>[154]</sup>. Expression of TcMCA3 was observed in all developmental stages, whereas that of TcMCA5 was limited to epimastigotes<sup>[155]</sup>. Two studies concluded that characterization of both MCAs might lead to discovery of potential novel drugs<sup>[156,157]</sup>. In addition, TcMCA3 regulation was dependent on its interaction with host procaspase-activating compound 1 (PAC-1), a procaspase 3 activator, that reduced mammalian cells viability<sup>[157]</sup>.

**Less studied CPs:** A new CP, named TcCPmet, was identified when expressed during *T. cruzi* metacyclogenesis. The investigators observed its inhibition by a broad spectrum CPI (E-64). It had a different elution pattern than that of CRZ, and was not recognized by anti-CRZ serum. It was able to hydrolyze peptides as CRZ, but at alkaline pH<sup>[158]</sup>. However, it was reported later that active proteases at acid pH are CPs, while those active at alkaline pH are metalloproteases<sup>[143]</sup>. For ATGs, a study demonstrated Atg4 and Atg8 over expression during differentiation of the developmental stages<sup>[159]</sup>.

### Cysteine protease inhibitors (CPIs)

All the investigated proteinase inhibitors (AMK, two DMK derivatives) demonstrated significant potentiality as anti-chagasic drugs. The first was the most efficient CRZ inhibitor, while DMKs showed no potency against epimastigotes<sup>[160]</sup>. The potential use of NO donors (NOR-3, SIN-1, SNAP, and SNP) was investigated in treatment of Chagas' disease and *falciparum* malaria. It was shown that NO donors inhibited CPs action *via* their cysteine catalytic residue<sup>[161]</sup>.

As previously reviewed<sup>[162]</sup>, peptide inhibitors gave *in vivo* unsatisfactory results due to their poor pharmacological profiles and their susceptibility to degradation by host CPs. Therefore, it was recommended to conduct structure-based screening

studies to identify new non-peptide and peptidomimetic inhibitors competent with CRZ crystal structure. Several studies utilized compounds containing nitrile group to be investigated as peptide CRZ inhibitors. Mexican investigators discussed structural features to improve the biological activity of nitrile group as potent peptide CRZ inhibitor<sup>[163]</sup>. Odanacatib (MK-0822), a drug containing nitrile group, was established as a potent reversible inhibitor for human CATH K. Sequence similarity between CRZ and CATH K encouraged Canadian investigators to evaluate odanacatib as CRZ inhibitor. They found that MK-0822 exhibited selective inhibitory potency against CRZ compared to human CATHs. Further optimization of odanacatib analogs enabled the investigators to identify two CPIs (Cz007 and Cz008) that showed both *in vitro* and *in vivo* efficacy in treatment of Chagas' disease. Besides, it was orally bioavailable, well tolerated, and with low cytotoxicity. Compound Cz007 in a daily dose of 3 mg/kg gave a 90% cure rate with 100% survival rate<sup>[164]</sup>. Followed by SAR studies, dipeptidyl nitrile scaffold were synthesized and investigated as potent CRZ inhibitors. For the SAR studies, the investigators assigned three, eleven and twelve substitutions at the P1, P2 and P3 positions of a dipeptidyl nitrile scaffold, respectively. The 23 lead inhibitors exhibited *in vitro* trypanocidal activity, however compound 12 was the most potent. Although no clear relationship was observed between CRZ inhibitory potency and trypanocidal activity, they claimed that such approach represented a valuable guide for identification of novel CRZ inhibitors for Chagas' disease treatment<sup>[165]</sup>.

Non-peptide CRZ inhibitors were investigated, the most common were semicarbazone derivatives. One of thiosemicarbazone derivatives combined with the aromatic protein of isatin scaffold was reported as potent CRZ inhibitor<sup>[166]</sup>. Novel series of semicarbazone and aldehyde-based non-peptide CRZ inhibitors were designed and synthesized in two American studies. The investigators demonstrated and validated utilizing SARs studies for lead compound optimization<sup>[167,168]</sup>. Similar results were obtained when Brazilian scientists investigated a series of thiosemicarbazone and semicarbazone derivatives as CRZ inhibitors<sup>[169]</sup>. Later, three other studies were conducted in Brazil for semicarbazone derivatives. The first study synthesized novel derivatives that proved to inhibit *T. cruzi* growth through an apoptotic pathway, not as CRZ inhibitor<sup>[170]</sup>. To optimize the activity of a thiosemicarbazone derivative, a 1,3-thiazole core was used in the second study, leading to identification of a potent CRZ inhibitor. Utilizing electron microscopy, compound (1c) showed irreversible and specific morphological alterations<sup>[171]</sup>. Series of semicarbazone, thiosemicarbazone, and aminoguanidine derivatives were synthesized and investigated as CRZ inhibitors. All derivatives demonstrated CRZ inhibition in a range of 70-75% with IC50 values against epimastigotes ranging from 20-140  $\mu$ M. Utilizing docking studies, the

investigators concluded that these derivatives were CRZ inhibitors attacking its cysteine residue, and they recommended further studies to validate optimization of such compounds<sup>[172]</sup>.

Vinyl sulfones also gained much attention as peptidomimetic CRZ inhibitors. Twenty years ago, K777 was utilized in treatment of Chagas' diseases, and it proved to clear parasitemia in chronically infected mice after 3 weeks of administration<sup>[173]</sup>. The same group of investigators evaluated the potentiality of *T. cruzi* to resist K777 administration for long time. They generated K777-resistant epimastigotes *in vitro* and found that resistance to 20-fold of its lethal concentration occurred after a year of gradual drug increase. Negligible CRZ expression was observed in resistant epimastigote extracts compared to the original control, and its activity was restored following drug withdrawal<sup>[174]</sup>. Later, the same inhibitor was used in *T. cruzi*-infected dogs and it prevented cardiomyopathy after a week of treatment<sup>[175]</sup>.

In their review aiming to discover new drug targets for treatment of Chagas' diseases, American scientists assigned two approaches. One of them had led to the discovery of K777 as a clinical drug candidate. They claimed that further improvement of K777 required searching for new scaffolds using molecular docking studies in screening ZINC database; the public library of commercially available compounds prepared especially for virtual screening<sup>[176]</sup>. Recently, all reports that utilized SAR studies after virtual or HTS were also reviewed. The reviewers concluded that non-peptide inhibitor; vinyl sulfone WRR-669 showed both *in vitro* and *in vivo* efficacy against Chagas' disease<sup>[177]</sup>.

A new strategy was proposed to design CRZ non-peptide inhibitor. Utilizing substrate activity screening (SAS), a new class of non-peptide triazole-based ketone inhibitors targeting CRZ was identified. High selective potency towards CRZ was improved on addition of a binding interaction in CRZ S3 pocket, i.e. incorporated with  $\beta$ -chloro vinyl sulfone pharmacophore. The new inhibitor showed complete eradication of *T. cruzi* from mammalian cell cultures<sup>[178]</sup>. Two years later, the same group of investigators utilizing CRZ high-resolution crystal structure confirmed its inhibition by this class of new inhibitors. Two intraperitoneal injections of a 20 mg/kg daily dose for 27 days revealed promising results in experimentally infected mice<sup>[179]</sup>.

Quinoxaline-N-acylhydrazone derivatives showed inhibitory potency against *T. cruzi* epimastigotes *in vitro*<sup>[180]</sup>. In a project to discover non-peptide CRZ inhibitors, the investigators utilized a combination of HTS, docking and co-crystallization studies to identify ML217. It exhibited *in vitro* trypanocidal activity with minimal cytotoxicity effects and *in vivo* efficacy in *T. cruzi*-infected mice<sup>[181]</sup>. Compounds WRR-483 and WRR-669 which are synthesized oxyguanidine analogs,

exhibited satisfactory results. The SAR studies confirmed the non-covalent binding of WRR-669 in CRZ crystal structure<sup>[182]</sup>. Computer-guided approach succeeded to identify clofazimine and benidipine with trypanocidal effects through their CRZ inhibitory potency. Results revealed that combined treatment reduced parasite burden in the cardiac muscle of chronically infected mice through a synergism mechanism<sup>[183]</sup>. Oxadiazoles were also suggested as potential scaffolds to be utilized in developing novel non-peptide CRZ inhibitors<sup>[184]</sup>.

Recently, a gene mutation in a non-active site of one of the genes encoding CRZ was observed. The investigators suggested that this discovery may open a new field of alternative inhibitor design<sup>[185]</sup>. Aiming to discover new scaffolds as CRZ inhibitors, Korean investigators utilized pharmacophore virtual screening, followed by molecular docking and molecular dynamics simulations. This was followed by SAR studies that specifically demonstrate CPI interactions with the catalytic residues to be locked at the S1, S2 and S3 subsites of CRZ. Utilizing this strategy, the investigators demonstrated that the two compounds selected as potential drugs for treatment of Chagas' disease were locked in CRZ active sites. Furthermore, both compounds recorded higher molecular dock scores and lower binding energies<sup>[186]</sup>.

#### Cystatins (CYsS)

Chagasin of *T. cruzi* was first isolated, immunolocalized and biochemically characterized by Monteiro *et al.*,<sup>[13]</sup>. Immunoblotting revealed that its expression was developmentally regulated and inversely correlated with CRZ expression. It was immunolocalized on the amastigote' surface and trypomastigotes flagellar pocket<sup>[13]</sup>. Variable expression levels were observed among six *T. cruzi* strains, with lower levels in avirulent isolates (i.e. poorly infective). The investigators concluded that chagasin indirectly modulated CRZ proteolytic functions, and played an essential role in epimastigote susceptibility to synthetic CPIs and their differentiation to metacyclic trypanosomes as well as *T. cruzi* virulence<sup>[187]</sup>. Interestingly, the non-infectivity of winged bugs by *T. cruzi* was attributed to CRZ modulation by tigut cystatin. The latter is the expressed CYS from the anterior midgut of *Triatoma infestans*<sup>[188]</sup>.

#### African trypanosomiasis

##### Cysteine proteinases (CPs) of *T. brucei* Cathepsins (CATHs)

In 1992, Kenyan investigators purified a CATH-L-like CP from bloodstream and metacyclic forms of *T. congolense*, and the term of trypanopain-*Tc* was raised. The enzyme showed proteolytic activity towards fibrinogen, serum albumin and trypanosome variant-surface glycoprotein (VSG)<sup>[189]</sup>. At the beginning of 20<sup>th</sup> century, the term rhodesain was introduced when American investigators purified it from *T. b. rhodesiense*. It was immunolocalized in the lysosome in all life cycle

stages. However, its expression was five times greater in short stumpy trypanosomes<sup>[190]</sup>. Two CATHs (B and L) were reported in *T. b. brucei* and *T. congolense*, and their corresponding CATHL was termed brucipain<sup>[191]</sup> and congopain<sup>[192]</sup>, respectively.

Utilizing gene knockout for both genes encoding CATHs showed that CATHB, not rhodesain played an essential role for *T. brucei* survival *in vitro*<sup>[193]</sup>. In spite of that, rhodesain (CATHL), rather than CATHB, was suggested as an appropriate drug target. Non-specific CATH inhibitors killed cultured parasites with >99% inhibition of both CATHs, while specific CATHB inhibitors did not affect parasite survival. In contrast, specific CATHL inhibitors killed parasites with >99% inhibition of CATHL, but only 70% CATHB inhibition<sup>[194]</sup>. Other functions were reported; expression of CATHB mediated degradation of host transferrin in the endosomal/lysosomal compartment<sup>[193,195]</sup>; while expression of CATHL of *T. b. gambiense* and *T. b. brucei* protected parasites *in vitro* against lysis<sup>[196-197]</sup>.

It is known that BBB is composed of brain microvascular endothelial cells (BMECs) especially designed to keep pathogens out. In their *in vitro* studies, the investigators observed that human-infective bloodstream forms of *T. b. gambiense* crossed BBB more efficiently than those derived from *T. b. brucei*-infected animals. Enhancement of cross barrier by *T. b. brucei* was observed if incubated with brucipain-rich supernatants expressed from *T. b. gambiense*. Accordingly, the investigators attributed BBB crossing in *T. b. gambiense* to the capability of brucipain to generate Ca<sup>2+</sup> activation signals in human brain microvascular endothelial cells. This is required for dynamics modulation and integrity of the endothelial cell monolayers leading to trans-endothelial migration through human BBB<sup>[198]</sup>.

#### Applications of CATHs in diagnosis and genotyping

During the period 2009-2014, several publications from Brazil reported utilizing CARHs for diagnosis and genotyping. A specific sequence in the gene encoding rangelpain, a CATH L-like CP from *T. rangeli*, showed homologous sequence in 17 isolates from humans and wild mammals. Rangelpain was validated as an excellent diagnostic marker for diagnosis and genotyping of *T. rangeli* by PCR<sup>[199]</sup>. Characterized sequences from genes encoding CATH L-like CPs from *T. vivax* isolated from African and South American cattle were investigated as biogenetic markers. Phylogenetic studies revealed two genotypes; cattle isolates from West Africa and South America belonged to *T. vivax* genotype, whereas cattle isolates from East Africa showed divergent sequences and belonged to *T. vivax*-like genotype<sup>[200]</sup>. Analysis of 78 CATHL catalytic domain sequences from 22 *T. theileri* isolated from cattle, water buffalo and deer identified six genotypes utilizing PCR. The investigators validated these sequences for population genotyping and evolutionary studies<sup>[201]</sup>.

The same group of investigators concluded usefulness of the CATHL catalytic domain sequences to identify two genotypes of *T. theileri* in cattle<sup>[202]</sup>. Similarly, *T. congolense* is a complex of three subgroups (Savannah, Forest and Kilifi) differing in virulence, pathogenicity, drug resistance, vectors, as well as geographical distribution. The same group of investigators compared sequences of genes encoding congopain from Savannah genome database and those of the three *T. congolense* subgroups. The obtained diverging results encouraged the investigators to develop PCR assay targeting congopain sequences to diagnose and differentiate between the three subgroups<sup>[203]</sup>.

### Calpains (CALPs)

Several genes encoding CALPs were identified in *T. brucei*. El-Sayed *et al.*<sup>[204]</sup>, in their bioinformatics analysis, pointed to the presence of a family of at least nine proteins (*TbCALP1.1-9*) with some degree of similarity to CALP proteolytic domain, but devoid of calcium-binding domains. Employing whole-genome analysis, a total of 18 CALP-like proteins were characterized in *T. brucei* and the investigators suggested their involvement in diverse cellular functions<sup>[6]</sup>.

An ortholog *TbCALP4.1*, also termed CALP5.5, was characterized as a cytoskeleton-associated protein exclusively detected in epimastigotes. The British investigators suggested that CALP5.5 was essential for cell morphogenesis<sup>[205]</sup>. Another British study reported stage-specific expression of CALP5.5 essential for *T. brucei* procyclic morphogenesis inside tsetse mid-gut<sup>[206]</sup>. A comprehensive analysis of the expression and transcription patterns of CALP-related proteins in *T. brucei* was conducted. It was found that a small kinetoplastid CALP-related protein (SKCRP5.1) and CALPs 8.1 and 5.5 were differentially expressed in trypanomastigotes, while transcripts of SKCRP1.6, SKCRP7.2 and CALPs 4.1 and 5.5 were in epimastigotes<sup>[207]</sup>. Later, SKCRPs 7.1 and 7.2 were identified in *T. brucei* under persistent endoplasmic reticulum stress. The investigators concluded the essential role played by SKCRPs to induce PCD in under-stressed parasites<sup>[208]</sup>.

### Metacaspases (MCAs)

Five genes encoding MCAs were identified in *T. brucei*, of which only MCAs 2 and 3 were expressed in the bloodstream forms, while MCA5 was expressed in the procyclic forms. It was found that only MCA5 possessed an additional C-terminal extension. Whereas MCAs 2, 3 and 5 possessed cysteine and histidine residues required for CP proteolytic activity, MCA1 and MCA4 lack both residues, and have a serine substitution instead of cysteine. Therefore, they lack usual cysteine catalytic activity<sup>[209]</sup>. On deletion of genes encoding MCA2 or MCA3 with MCA5 ( $\Delta mca2/3\Delta mca5$ ), the investigators observed successful growth of mutant parasites *in vitro* and *in vivo*. It was attributed to

compensative overlap function of MCAs 2 and 3. There was no change in the susceptibility of *T. brucei* to stress, similar to WT. On simultaneous deletion of all genes, immediate growth arrest was observed. The investigators recommended further studies to elucidate roles of MCAs in PCD machinery pathway in *T. brucei*<sup>[210]</sup>. In a British study, recombinant *T. brucei* MCA 2 (*TbMCA2*) showed calcium-dependent proteolytic activity. It was claimed that *TbMCA2* activity was different from that of caspases suggesting its different physiological roles in *T. brucei*<sup>[211]</sup>.

During the period, 2012-2017, a team of scientists from Brazil and UK published three reports. The crystal structure of *TbMCA2* was determined and revealed procession of an unusual N terminus that regulated substrate access to the active site. In the presence of its specific substrate, *TbMCA2* was activated by calcium. Utilizing phylogenetic analysis, the investigators suggested that caspases and *TbMCAs* evolved from a common ancestor, but independently showed distinct activation mechanisms to regulate PCD pathways. Accordingly, the investigators recommended future studies to design MCA specific inhibitors for development of novel anti-protozoal drugs<sup>[212]</sup>. In the second report which utilized *TbMCA2* crystal structure, four observations were demonstrated; 1) strict preference for charged basic amino acid, e.g. arginine or lysine at the P1 position; 2) non-basic residues at P1 were competitive *TbMCA2* inhibitors; 3) negative charged residues, e.g. asparagine and glutathione at P2 and P3 positions were resistant to *TbMCA2* hydrolysis, and 4) two Ca<sup>2+</sup>-binding sites with reversible structural modification were close to the S2 binding pocket upon Ca<sup>2+</sup> activation. Interestingly, one Ca<sup>2+</sup>-binding site was localized on the surface of asparagine residue (Asp<sup>173</sup>, Asp<sup>189</sup>, Asp<sup>190</sup> and Asp<sup>220</sup>), a location distinct from both the catalytic active site and the S2 binding pocket<sup>[10]</sup>. The third article reported that *TbMCA2* was active without processing, and cleavages at lysine<sup>55</sup> and lysine<sup>268</sup> increased its activity on synthetic substrates<sup>[213]</sup>. Recently, South African investigators biochemically characterized *T. congolense* MCA5 aiming to develop novel drugs for treatment of livestock trypanosomiasis. Similar results were obtained for substrate preference and Ca<sup>2+</sup>-binding prior to activation, as previously reported for *TbMCA2*<sup>[214]</sup>.

Based on the previous mention that *T. brucei* MCAs 1 and 4 had active site substitutions and lacked proteolytic activity, a study reported *TbMCA4* as virulence factor. Parasites mutant in gene encoding *TbMCA4* showed normal growth *in vitro*, but significant reduced virulence *in vivo*. It was demonstrated that MCA4 was expressed only in bloodstream forms due to its specific processing by MCA3, i.e. MCA4 requires MCA3 to be processed as proteolytic enzyme. It was concluded that *TbMCA4* was a pseudo-peptidase, but it was linked as virulence factor due to its association with MCA3 in its proteolytic cascade. Future studies

were recommended to investigate MCA4 role in natural chronic infections<sup>[215]</sup>.

### Cysteine proteinase inhibitors (CPIs)

Several compounds were investigated as anti-trypanosomal chemotherapeutic agents through their inhibitory effects on brucipain. These included non-peptide<sup>[216-221]</sup> and peptidomimetic<sup>[222-224]</sup> inhibitors.

**Non-peptide inhibitors:** Quantitative HTS approach utilized to search for a CRZ inhibitor identified new compounds of triazine nitriles. Three studies were conducted in USA. In the first study, structural scaffold modifications that improved their *in vitro* potency against both CRZ and rhodesain were performed. The selected lead compounds that showed higher selectivity to CRZ and rhodesain than human CATHs, also showed significant inhibitory potency against *T. brucei in vitro*<sup>[216]</sup>. Oral administration of one of the lead compounds showed significant increase of survival rate and time in *T. b. brucei*-infected mice, but with a little effect on *T. b. rhodesiense*-infected mice. Moreover, it showed high potency to cross BBB<sup>[217]</sup>. Utilizing SAR analyses of the lead triazine nitriles, the investigators were able to identify the binding preferences of CRZ and rhodesain active site, individually; i.e. its S2 pocket. Imidazopyridine nitrile showed significant stability and selectivity toward the active site of both CPs. Moreover, it showed a fourfold lower cytotoxicity than the parent triazine nitrile<sup>[218]</sup>.

Dipeptide nitriles, a new class of potent non-peptide CPIs against papain-like proteases were investigated. The investigators synthesized compounds that showed inhibitory potency against recombinant CPs of *T. brucei* (rhodesain and CATHB) as well as CRZ. They succeeded to generate activity-based probes serving as tools for further development of synthesized azanitriles as potential anti-trypanosomal drugs<sup>[219]</sup>. Later, a series of dipeptide nitriles known to inhibit mammalian CATHs were investigated against rhodesain. Based on SAR analyses, the investigators observed that compound 35 possessed a leucine residue and a phenyl ring to fit into S2 and S3 pockets of rhodesain, respectively<sup>[220]</sup>. On the other hand, the inhibitory activity of niacin, one of nicotinamide derivatives, on *T. brucei* growth was investigated. Electron microscopy revealed severe defects in endocytic traffic with enlargement of the flagellar pocket, and lysosomal disruption ended with trypanosomes death. A direct inhibitory activity assay of recombinant *TbCATHB* confirmed its mechanism of action as CPI. Due to several reasons including being cheaply produced, oral administration, BBB crossing, and interference with trypanosomes iron metabolism, nicotinamide derivatives were suggested as a new class of anti-trypanosomal drugs<sup>[221]</sup>.

**Peptidomimetic inhibitors:** It was found that vinyl sulfones had a potentiality to serve as Michael acceptors for the nucleophilic active site cysteine. The investigators recommended further studies to

synthesize second generation of vinyl sulfones<sup>[222]</sup>. Utilizing structure-guided approach, two new classes of synthesized vinyl sulfones were identified, and their IC50 against rhodesain, *TbCATHB* and CRZ were determined. Results revealed that analogs 7 and 8 targeted S2 and S3 of all investigated CATHs. When evaluated *in vitro*, they showed efficient potency against *T. b. brucei*, without significant cytotoxicity effects<sup>[223]</sup>. The efficacy of K11777, combined with current drugs used for African trypanosomiasis, such as suramin, pentamidine, melarsoprol and eflornithine, on *T. brucei* bloodstream forms *in vitro*, was investigated. A synergistic effect was observed after treatment of K11777 with eflornithine, in contrast to antagonistic effects shown with the other drugs<sup>[224]</sup>.

### Cystatins (CYsS)

To investigate the role played by *T. brucei* chagasin, a group of scientists from Brazil and UK generated chagasin-mutant bloodstream forms. Function regulation of *T. brucei* CPs was suggested through modulating surface coat exchange during differentiation, degrading IgG against VSGs, and immunomodulating host immune response, thus increasing its infectivity and virulence<sup>[225]</sup>.

## CONCLUDING REMARKS

1. There are three CPs (CATHs, CALPs and MCAs) and only one endogenous CPI (chagasin) in pathogenic trypanosomatids: *Leishmania* spp., *T. cruzi* and *T. brucei*. All CATHs possess a long C-terminus extension (CTE), and they play crucial roles in parasite virulence, and elicit host immune response. With special emphasis on CRZ, all genes encoding CPs were investigated as drug targets, diagnostic and biogenetic markers, as well as vaccine candidates.
2. Genomic analysis identified several genes encoding CALPs with significant potentiality as drug targets. A total of 27, 24 and 18 CALP-like proteins were characterized in *L. major*, *T. cruzi* and *T. brucei*, respectively. According to their structure, small kinetoplastid CALP-related proteins (SKCRPs) possess an exclusive N-terminus prodomain. It was reported that *T. brucei* possessed several SKCRPs, differentially expressed in all its developmental stages under persistent endoplasmic reticulum stress.
3. An important difference was reported for all trypanosomatids MCAs; they require calcium prior to activation. No evidence was shown to be involved in PCD, all studies concluded that their role in autophagy is essentially for proliferation and differentiation.
4. *L. donovani* and *T. cruzi* possess two MCAs. In *T. cruzi*, gene encoding MCA3 is composed of 16 copies, while a single copy encoding MCA5 was observed. Although five MCAs were identified in *T. brucei*, only MCAs 2, 3 and 5 possessed cysteine and histidine residues required for CP proteolytic

activity. On the other hand, MCA1 and MCA4 lack both residues, with a serine substitution instead of cysteine, hence they lack usual cysteine catalytic activity.

5. Chagasin has unique immunoglobulin fold, acting as scaffold for three loops of highly conserved sequences. It has a specific mode of interaction with CPs that is different from other CYSSs previously reported in parasites. Hence, it is placed by MEROPS website in a recent classification as clan IX, family I42. Host-parasite interaction *via* modulating host immune response and host CPs activity was suggested as a main role for chagasin.
6. *Leishmania* spp. include three CATHs: two L-like (CPA and CPB) and one B-like (CPC). A single copy of each gene encoding CPA and CPC were identified, while that encoding CPB is a multi-copy gene located in a single locus and arranged in a tandem repeat. It was reported that gene expression of copy *cpb2* was mainly in metacyclic forms, and those of last 16 copies (*cpb3-cpb18*) were in amastigotes. The copy *cpb2.8* without its CTE was suggested as an immunomodulator of Th1/Th2 immune response.
7. Because man is infected by several *Leishmania* spp., CATHs expression was attributed for variable clinical presentations in CL and VL, with specific emphasis on CATH B. The latter was also confirmed as virulence factor due to its essential roles in parasite growth, viability, autophagy, differentiation and host cell invasion.
8. Several genes encoding CRZ were reported giving rise to several isoforms with varying degrees of genetic diversity. It was immunolocalized in lysosomes of amastigotes and trypomastigotes and reservosomes of epimastigotes. Beside its role in *T. cruzi* survival, egress and virulence, its expression was responsible for induction of strong humoral and cellular immune responses resulting in cardiological manifestations associated with Chagas' disease. Its domain possesses unusual sulfate groups in its N terminus extension that proved to immunomodulate host immune response.
9. Brucipain, rhodesain and congopain are terms used for CATH L-like CPs of *T. b. brucei*, *T. b. rhodesiense*, and *T. congolense*, respectively. It was suggested that crossing of *T. b. brucei* to BBB was attributed to brucipain capability to generate Ca<sup>2+</sup> activation signals in human brains, required for dynamics modulation and integrity of the endothelial cell monolayers. Also, congopain gained much attention due to its essential roles in cattle trypanosomiasis as it was widely investigated as vaccine candidate.
10. Several compounds, investigated as CPIs, proved high potency against *Leishmania* CATHs, CRZ and rhodesain or its analogs. However, vinyl sulfones, semicarbazone derivatives and triazole-based ketone inhibitors gained much attention. To design novel non-peptide CPIs, several studies documented utilizing a combination of virtual

screening or HTS, docking studies and SAR analysis using target CP crystal structure.

**Conflict of interest:** There is no conflict of interest.

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