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Trypanosoma cruzi Peptidases: An Overview

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Abstract: Peptidases are a group of enzymes which have a catalytic function that is to hydrolyze peptide bonds of proteins. The enzymes that hydrolyze peptide bonds at the amino- or carboxy- terminus are classified as exopeptidases, and those that cleave peptide bonds inside the polypeptide are endopeptidases. Endopeptidases, such as cysteine-, metalo-, serine- and threonine peptidases as well as some exopeptidases, have been characterized in *Trypanosoma cruzi*. Understanding the pathogenesis of *T. cruzi* requires the identification of functional properties of those peptidases, as they are implied in virulence, are important for host-parasite interactions and are critical for successful survival in their hosts. Here we examine the main *T. cruzi* peptidases, focusing on their biological roles, especially concerning the parasite-mammalian host relations.

Keywords: Peptidases, *Trypanosoma cruzi*.

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas disease, also known as American trypanosomiasis, preferentially infects the heart leading to cardiac arrest, which is frequently followed by death [1]. Chagas disease is among the thirteen parasitic tropical infections that are considered to have the highest-burden of the neglected tropical diseases, which together affect more than one billion people worldwide [2,3]. They are largely ignored by medical science, first-world public opinion and pharmaceutical companies [4, 5]. *T. cruzi*, a flagellated protozoan parasite, has a complex life cycle, including two replicative forms: the epimastigote, present in the gut of the insect vector, and the amastigote, an intracellular form in the infected mammal. The two infective non-replicative forms are the metacyclic trypomastigote in the insect vector and the bloodstream trypomastigote released from infected cells into the blood of the mammal. The intracellular lifestyle of *T. cruzi* enables the parasite to evade host defenses and results in the chronic disease stage. Eventually, after years of asymptomatic infection, 15–30% of patients develop signs of organ damage, which produces characteristic cardiac, digestive or nervous forms of chronic Chagas disease; death is a frequent outcome [6].

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The World Health Organization has estimated that some 16–18 million people are infected throughout the American continent, including 100,000 in the United States, and 120 million people are at risk of infection throughout Central and South America [7]. Two clinical forms are characteristic of this disease, acute and chronic. In the acute phase, a local inflammatory lesion appears at the site where metacyclic trypomastigotes enter, and the parasite spreads throughout the host organism. The presence of myocarditis and/or pathological disturbances in the peripheral nervous system and gastrointestinal system are features observed in the chronic phase. Thirty to forty percent of chronically infected individuals develop cardiac abnormalities and as many as 10% develop digestive tract diseases [8]. To date, no vaccines have been developed, and there are no prophylactic drugs to prevent infection by *T. cruzi*. Moreover, current available medication, such as nitrofurans (nifurtimox) and nitroimidazoles (benznidazole), are effective during the acute phase of the infection but have little effect in the chronic stage of the disease [9]. In addition, both drugs have serious side effects and resistance against both compounds leads to increasing treatment failures [10]. Taking into account that benznidazole and nifurtimox are considered far from ideal as trypanocidal drugs, the search for new compounds with anti-*T. cruzi* activity, low toxicity and increased efficacy during the chronic phase continues. In the last few years, progress towards new drugs for the treatment of Chagas disease has been disappointing. Chemotherapy still relies on drugs developed decades ago showing limited efficiency and toxic side effects [11, 10]. Efforts to tackle this disease require

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research on host-parasite interactions, especially the initial events, which may lead to the establishment of the disease and determining when many different parasite molecules are known to be involved [12, 13]. Some headway has been made in identifying targets that are key to parasite virulence and the pathogenesis of the disease. Among the most promising targets for antiparasitic agents are parasite peptidases in order to establish novel, effective and selective chemotherapies. These enzymes play central roles in diverse processes, such as adhesion and cell invasion, quite apart from their obvious participation in the nutrition of the parasite at the expense of the host [14].

Taking into consideration the importance of *T. cruzi* peptidases in the development of Chagas disease and the fact that they have been described and characterized as promising drug targets [15], this review will primarily focus on our current knowledge of the role of peptidases in virulence, pathogenicity and the development of peptidase inhibitors as potential drugs to treat Chagas disease.

PEPTIDASES

Peptidases, proteases or proteolytic enzymes are hydrolytic enzymes that cleave peptide bonds in proteins. The MEROPS database is an information resource for peptidases and includes their classification, structure and properties [16-18]. Endopeptidases, such as cysteine-, metallo-, serine- and threonine-peptidases as well some exopeptidases, have been characterized in *T. cruzi*. The recent completion of the genome sequence of *T. cruzi* (CL Brener clone) suggests the presence of 70 putative cysteine peptidases, about 40 putative serine peptidases, 250 putative metallo-peptidases (of which most are homologues to leishmanolysin (Gp63), 25 putative threonine peptidases (most of which are homologues of proteasome Subunits), and 2 putative aspartyl peptidases. It is noteworthy that among the latter, the complete absence of A1 family peptidases (pepsin-like) has been reported for the genomes of the three trypanosomatids analyzed: *T. cruzi*, *Leishmania major* and *Trypanosoma brucei* [19]. The genes detected in *T. cruzi* were putative presenilin-

Table 1. Major *Trypanosoma cruzi* Peptidases

Catalytic type	Peptidase Name	Endo-Exopeptidase	Clan	Family	NC-IUBMB	MEROPS ID
Cysteine	Cruzipain (CZP)	Endo	CA	C1	3.4.22.51	C01.075
	Cathepsin B-like cysteine endopeptidase	Endo	CA	C1	*	C01.098
	Cruzipain 2	Endo	CA	C1	*	C01.100
	Metacaspase 9	Endo	CD	C14	*	C14.034
	Calpain	Endo	CA	C2	*	C02.024
Serine	Oligopeptidase B (Tc 80)	Endo	SC	S9	3.4.21.83	S09.010
	Prolyl oligopeptidase (POP)	Endo	SC	S9	3.4.21.26	S09.001
Metallo	Metallopeptidases (carboxypeptidase)	Exo	MA	M32	*	M32.004
	GP63	Endo	MA	M8	3.4.24.36	M08.001
Threonine	Proteasome	Endo	PB	T1	3.4.25.1	(subunit 1) T01.010 (subunit 2) T01.011 subunit 3 T01.012 (subunit alpha 2) T01.972 (subunit alpha 7) T01.974 (subunit alpha 5) T01.975 (subunit alpha 1) T01.976
					*	T01.021

*Not yet included in IUBMB.

like aspartic peptidase, Clan AD, family A22A and putative signal peptide peptidase, aspartic peptidase, Clan AD, family A22B [20, 21]. However, recently two aspartyl peptidases were identified in *T. cruzi* by Pinho [22]. In the aspartyl peptidases section, this question will be briefly discussed.

Several functions had been described for peptidases in *T. cruzi*, including virulence factors. Besides this, they are important enzymes for host-parasite interactions, and they are critical for successful parasite survival [23]. Table **1** summarizes the major *T. cruzi* peptidases.

CYSTEINE PEPTIDASES

Cysteine peptidases present a catalytic cysteine residue, which mediates protein hydrolysis *via* a nucleophilic attack on the carbonyl carbon of a susceptible peptide bond [24-26]. A closely related family of cysteine peptidases, known as cruzipain (CZP), cruzain (the recombinant catalytic domain of cruzipain), or GP57/51, constitutes the major cysteine peptidase activity in *T. cruzi* [9, 27, 28]. Another cysteine found in this parasite is a 30-kDa cathepsin B-like cysteine peptidase [29]. The occurrence of Metacaspases [21], and recently the Calpains, have also been described in this parasite [30, 31].

Cruzipain

Cruzipain is encoded by a family of genes organized as a series of tandem repeats located on different chromosomes. It is expressed as a complex mixture of isoforms in the main developmental stages throughout the *T. cruzi* life-cycle, i.e., in the extracellular trypomastigote, amastigote and insect epimastigote stages. Activity levels are highest in epimastigotes, and depending on the life cycle stage, cruzipain is found on the cell surface of *T. cruzi* amastigotes or lysosome-like organelles present on *T. cruzi* epimastigotes called reservosomes [32-34].

Like the mammalian enzyme, the trypanosome peptidases are synthesized as pre-pro-proteins [14, 35]. This enzyme, with a molecular mass ranging from 40 to 60 kDa, is an endopeptidase able to digest several protein substrates including casein, bovine serum albumin, denatured hemoglobin and also small peptides [24]. Duschak and collaborators [36] reported a novel cysteine secreted by metacyclic trypomastigotes, named TcCPmet, migrating in SDS-gelatin at 98-116 kDa. This novel proteolytic activity did not react with anti-cruzipain antibodies, suggesting that this extracellular enzyme is not a cruzipain homologue.

In addition to their role in parasite nutrition as its major lysosomal peptidase, the cysteine peptidases have been implicated in a number of cellular processes including cell invasion, proliferation, parasite differentiation and metacyclogenesis. Recent studies demonstrated that tissue culture trypomastigotes generate kinins through mechanisms that involve cooperative interactions between cruzipain, high molecular weight kininogens (HK) and heparan sulfate proteoglycans [1, 37], as described in the section "Role of peptidases in *T. cruzi* interaction" of this review. It is also an immunodominant antigen recognized during human infection, with a possible participation in the defense of the parasite against the immune system of the mammalian host [1, 38].

Cathepsin B-Like Cysteine Endopeptidase

In addition to cruzipain, which shows homology with cathepsin L peptidase, *T. cruzi* life-cycle forms express cathepsin B, a peptidase considered a virulence factor, as evidenced by its overexpression in a parasite stock resistant to cruzipain inhibitor. The enzyme has been purified and shown to be an acidic cysteine peptidase of 30 kDa with broad substrate specificity [29, 39, 40]. Sera from both chagasic rabbits and humans have specific antibodies to highly purified native oligopeptidase B and cathepsin B. The antibody levels to cathepsin B were higher than those observed to oligopeptidase B. Sera from mucocutaneous leishmaniasis and kala-azar patients have antibodies that recognize cathepsin B as an antigen, showing cross reactivity. Despite high levels of specific antibodies, sera from *T. cruzi*-infected patients did not inhibit cathepsin B activity. One possible function for both oligopeptidase B and cathepsin B, associated with Chagas disease pathogenesis would be to hydrolyze host proteins and induce host immune responses after enzyme release upon parasite lysis [41].

Metacaspases

The caspases are a family of cysteine peptidases that are the main effectors of apoptosis or programmed cell death [42]. Until recently, it was thought that lower eukaryotes might lack caspases because no homologous caspase genes had been discovered in the yeast genome or in the genome of any parasitic protozoa. Recently, the genome of *T. cruzi* CL Brener was shown to contain two genes, TcMCA3 and TcMCA5, with homology to those encoding metacaspases. TcMCA3 is expressed in the four major developmental stages of the parasite, whereas TcMCA5 is expressed only in the epimastigote form [20]. Experimental evidences suggest that the metacaspases might be involved in programmed cell death (PCD) of the parasite [21]. Caspase-like protein activity was also detected in epimastigotes of *T. cruzi* maintained in axenic cultures [43].

Caspase-like activity was detected in parasites at exponential and stationary phases of growth using acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), a caspase-3 specific substrate. At the stationary phase of growth, caspase 3-like activity increases, suggesting that this type of cysteine-protease could be involved in apoptosis-like cell death observed in the parasites. The presence of caspase 3-like proteins was confirmed by Western Blot using polyclonal heterologous antibodies against human procaspase 3, both in *T. cruzi* Tulahuen and MF strains [43], presenting the expected molecular mass for *T. cruzi* metacaspase 3 found by Kosec [21].

Calpains

Calpains are calcium-dependent cysteine peptidases involved in several physiological functions including apoptosis, signal transduction, cell differentiation and cytoskeleton remodeling [44, 30]. In humans, they exist in two major isoforms, m-calpain and μ-calpain, which require millimolar and micromolar concentrations of Ca^{2+}, respectively, for their activation [31]. The gene encoding the *T. cruzi* CALP, TcCALPx11 (GeneDB ID Tc00.1047053506563.210), was identified by microarray analysis as being differentially expressed in *T. cruzi* during metacyclogenesis. Recently, a cal-

pain inhibitor III (MDL28170) was used to detect calpain-like molecules in epimastigote forms of the *T. cruzi* isolate Dm28c. A 80 kDa reactive protein was detected by Western blotting [30]. Different levels of CALPs expression were also detected in distinct phylogenetic lineages, such as Y strain (lineage TCI), Dm28c (TCII) and INPA6147strain (Z3 zymodeme). Addition of 70 mM of MDL 28170 to the culture medium promoted a strong reduction in the growth rate of epimastigote forms of the parasite suggesting an *in vivo* relevance. [31].

SERINE PEPTIDASES

The prolyl oligopeptidase family cannot hydrolyze peptides containing more than about 30 residues. This group is unrelated to the classical trypsin and subtilisin families and includes dipeptidyl peptidase IV, acylaminoacyl peptidase and oligopeptidase B, in addition to the prototype prolyl oligopeptidase. In trypanosomatids, serine peptidase research has centered on the Clan SC peptidases, oligopeptidase B (OpdB) and prolyl oligopeptidase (POP) [45]. *T. cruzi*-host cell invasion studies have shown that oligopeptidase B generates an active Ca^{2+}-agonist from a cytosolic precursor molecule. Deletion of OpdB impairs the ability of *T. cruzi* to invade host cells and attenuates virulence *in vivo* [12, 46, 47]. Antibodies to the recombinant oligopeptidase B inhibited both peptidase activity and Ca^{2+}-signaling [46]. *T. cruzi* prolyl oligopeptidases (POP) specifically hydrolyze human collagen (Types I and IV) and fibronectin and have been implicated in the parasite adhesion to host cells and cell entry [48,49]. Relatively less work has been focused on chemotherapeutic hits or leads against protozoan serine peptidases, but interest in protozoan subtilisin-like targets is increasing. The invasive capacity of *T. cruzi* is reduced *in vitro* in the presence of OpdB and POP inhibitors [45, 48, 50].

T. cruzi also has shown serine carboxypeptidase activity belonging to the C group of serine carboxypeptidases, within the S10 serine peptidase family, which shows a high similarity to plant and yeast enzymes [51]. The enzyme is a monomeric glycoprotein with a molecular mass of 54 kDa, and is highly specific for hydrophobic C-terminal amino acid residues, and is strongly inhibited by 3,4-dichloroisocoumarin. Recently, it was demonstrated that this proteolytic activity is present in lysosome-related organelles as cruzipain [34].

METALLOPEPTIDASES

In recent years, the number of identified metallopeptidases has increased dramatically. In the Trypanosomatidae family, these enzymes play important roles in nutrition, pathogenicity, survival and interaction with the host [24]. Metallopeptidase activities have been described in several monogenetic and digenetic trypanosomatids [52-54], but only the so-called gp63 from *Leishmania* spp. has been thoroughly characterized. Genes homologous to gp63 of *Leishmania* are also present in this parasite. Four gp63 homologous genes in *T. cruzi* are differentially expressed, being more abundant in amastigotes than in epimastigotes or trypomastigotes [55].

In *T. cruzi*, different metallopeptidase activities have been previously described, some of them expressed specifically during metacyclogenesis in various strains and clones of *T. cruzi* [56, 57]. In addition, Cuevas [58] described that *T. cruzi* possesses a family of gp63 genes composed of multiple groups. Two of the groups, *Tcgp63*-I and *Tcgp63*-II, are present as high-copy-number genes and antibodies against *Tcgp63*-I partially blocked the infection of Vero cells by trypomastigotes, which suggests a possible role for this metallopeptidase during the infection process *in vitro*.

Matrix metallopeptidases (MMPs) are a family of structurally related zinc-dependent peptidases that are thought to be responsible for normal matrix remodeling and pathological tissue destruction by virtue of their ability to hydrolyze the major components of the extracellular matrix (ECM) [59].

Earlier studies from our laboratory showed that MMP-9-like activity was detected in the cytoplasm of *T. cruzi* during *in vitro* infection of embryonic hepatocyte cells [60]. Peptidase-dependent extracellular matrix remodeling is one of the events that it is emerging as a key regulator of *T. cruzi* infection and the pathogenesis of Chagas disease. So, the presence of MMP-9-like activity in *T. cruzi* may contribute to ECM protein degradation facilitating invasion of host cells, an activity that is likely to be highly relevant *in vivo* during the navigation of interstitial tissue spaces by trypomastigote forms [61]. Recently, it has been shown that the expression and activity of two MMPs, MMP-2 and MMP-9, are upregulated in cardiac tissue during the acute phase of *T. cruzi* infection and they are detected in association with inflammatory cells infiltrating the myocardium [62]. Additionally, in order to establish the role of MMPs *in vivo*, *T. cruzi*–infected mice were treated with doxycycline, a potent inhibitor of MMP activity. It was found that mice treated with doxycycline showed significantly decreased heart inflammation, delayed peak of parasitemia and improved survival rates, compared with the control group. This evidence suggests an important role for MMPs in the induction of *T. cruzi*-induced acute myocarditis. Furthermore, our group has demonstrated for the first time MMP-9-like activity in *T. cruzi* [63]. Gutierrez *et al* [62] showed that MMP-9 expression is upregulated during *T. cruzi* infection.

The study of exopeptidases in *T. cruzi*, on the other hand, has received little attention. Two metallocarboxypeptidases (MCPs) of the M32 family from *T. cruzi* CL Brener clone have recently been characterized: *Tc*MCP-1 and *Tc*MCP-2 [64]. The structure of *Tc*MCP-1 shows strong topological similarity with archaeal, bacterial and mammalian metallopeptidases including angiotensin-converting enzyme, neurolysin and thimet oligopeptidase [65].

Thus, *Tc*MCPs might be involved in the degradation of peptides, such as those produced by the proteasomes, in the cytosol. However, a possible role in the processing of proteins and small regulatory peptides cannot be excluded. These are the first members of this family to be characterized in a eukaryotic organism, and these peptidases may be a new target for the development of a rational chemotherapy against Chagas disease and other diseases caused by trypanosomatids.

PROTEASOME

In eukaryotic cells, the turnover of intracellular proteins is mediated mainly by the machinery of the ubiquitin/adenosine-5'-triphosphate (ATP)-dependent protea-

some pathway, which is a strictly controlled enzymatic complex [66, 67]. The mechanism of degradation is successive: protein substrates selected for destruction are tagged by the covalent addition of poly-ubiquitin chains (E1-E3 ubiquitin conjugation pathway), which are recognized and proteolytically degraded by the multifunctional 26S proteasome complex, a large non-lysosomal multi-subunit and multi-catalytic peptidase complex located in the cytosolic and the nuclear compartments [68]. This proteolytic pathway controls a broad array of cellular functions, including stage-specific gene transcription [69], antigen-processing [70], cell cycle progression [71], regulation of membrane-anchored and secretory pathway-compartmentalized proteins [72] and protein quality control [73].

The functional proteasome (known as 26S proteasome) is an ATP-dependent, multifunctional proteolytic complex that differs in many respects from typical proteolytic enzymes [74]. It consists of a proteolytic core particle, the 20S (720 kDa) proteasome, capped at one or both ends by a regulatory component termed the 19/22S complex (890 kDa) (regulatory particle), also called the proteasome activator (PA)700, composed of at least 18 peptides that determine substrates specificity for the selective degradation of ubiquitinated proteins [75]. Ubiquitinated substrates are processed at the active sites located within the inner cavity of the core particle, whereas the regulatory particle is responsible for recognition, unfolding and translocation of the selected substrates into the lumen of the core particle.

Mammalian cells and the protozoan *T. brucei* also contain an ATP-independent activator of the 20S proteasome, named the PA28 or PA26 complex, respectively, that can replace 19S and activates the proteolysis of short peptides. The PA28 activator is a 200 kDa ring-shaped heteromultimer composed of two isoforms of a 28 kDa subunit (PA28α and PA28β) and is present in the cytoplasm as a free complex or associated with the proteasome. Crystallographic studies of the 20S proteasome revealed a complex cylindrical structure made up of four stacked heptameric rings composed of seven different α-subunits in the outer rings and seven different β-subunits in the inner ones. The active sites reside within the β-subunits, which provide the catalytic N-terminal threonine residues. The eukaryotic 20S proteasome shows several distinct proteolytic activities, since *in vivo* assays with chromogenic substrates demonstrated the following activities: a chymotrypsin-like activity, a trypsin-like activity, a peptidyl-glutamyl-peptide-hydrolyzing activity, a branched chain amino acid-preferring activity and a small neutral amino acid-preferring activity. However, the chymotrypsin-like activity and the trypsin-like activity are considered the main activities [76].

The presence of the 26S proteasome and the ubiquitin pathway in *T. cruzi* was documented for the first time by De Diego [77]. The 26S proteasome of *T. cruzi* epimastigotes was identified as a high molecular mass complex (1400 kDa) with a composition that resembles that of the 26S proteasome isolated from other eukaryotic cells and with an ATP-dependent chymotrypsin-like activity against the substrate Suc-Leu-Leu-Val-Tyr-AMC (Suc corresponding to Succinyl, AMC being 7-amino-4-methylcoumarin). Some members of the highly conserved AAA family of ATPases present in the 19S complexes that cap the 26S proteasomes were also identified [78]. The characterization of the proteasomes from trypanosomes and their comparison with rat proteasomes has shown that they share structural similarities but are functionally distinct [79]. Therefore, it would be of great interest to investigate the subunit composition of trypanosome proteasomes further and to compare it with that of proteasomes from other organisms, as well as to isolate genes encoding proteasomal subunits. Two genes encoding *T. cruzi* proteasome subunits α_1 [80] and α_6 [81] were cloned and characterized and the results showed that the *T. cruzi* proteasome might be composed of the same set of subunits as other eukaryotes. However, it is still not clear whether all of the corresponding α- and β- family subunits are found in the proteasomes of early diverging eukaryotes, such as *T. cruzi*.

Experiments with specific inhibitors of proteasome activity such as peptide aldehydes (e.g. N-carbobenzoxyl-Leu-Leu-leucinal, MG132), lactacystin and gliotoxin have helped to define the role of the proteasome in various parasitic protozoa processes, including replication and differentiation [82]. Proteasome involvement in the stage-specific trypomastigote-amastigote transformation has been a special focus of attention in *T. cruzi*. Since this parasite undergoes shape changes, genes must be turned on and off and the proteins must be selectively degraded and synthesized [83].

T. cruzi treatment with lactacystin inhibits both the transformation of trypomastigotes into amastigotes and the development of amastigotes into trypomastigotes, thus implicating proteasomes in parasite remodeling [84]. Moreover, lactacystin treatment promotes the conjugation of ubiquitin to flagellar proteins and the accumulation of ubiquitinated products during transformation [77, 84]. It seems that proteasome activity is not required for host cell invasion since lactacystin pre-treatment of trypomastigotes (10 μM) caused no effect on the number of intracellular parasites in cultured myoblasts [82]. Recently, Cardoso [85] found that epimastigotes treated with lactacystin were still able to adhere to the culture flask walls, but did not differentiate into metacyclic trypomastigotes. In addition, lactacystin treatment blocked epimastigote multiplication, since flow cytometry data demonstrated that *T. cruzi* epimastigotes cultured in lactacystin-containing LIT medium were arrested at the G2 phase of the cell cycle. These findings suggest that proteasomes are involved in *T. cruzi* cell growth and metacyclogenesis *in vitro*.

Antigenic peptides derived from intracellular proteins are continuously presented to the immune system by MHC class I molecules on the surface of immune and non-immune cells, and the major proteolytic system generating peptide ligands are dependent on proteasomes. The 20S proteasome is the key peptidase generating peptides for the MHC class I antigen presentation pathway. In this context, studies have been done in order to answer whether *in vitro T. cruzi* infection might influence the gene expression or protein profile of the 20S proteasome [86].

ASPARTIC PEPTIDASES

Aspartic peptidases are a relatively small group of proteins that have received enormous interest because of their significant roles in human diseases. In *T. cruzi* (epimastigote forms), two aspartic peptidases have been isolated and iden-

tified as cruzipsin-I (CZP-I) and cruzipsin-II (CZP-II). Both peptidases show a molecular mass of 120 kDa by HPLC gel filtration. By the substrate-sodium dodecyl sulphate-polyacrylamide-gelatin gel electrophoresis method, it was possible to detect the activity of the enzymes in a doublet of bands at 56 and 48 kDa [87,89]. Nevertheless, due to large difficulty to correlate homologous genes by computer techniques of both peptidases, there are still several sequences that have not been correctly identified [19, 20, 21]. Moreover, the physiological role of these *T. cruzi* peptidases has not been completely understood [87].

ROLE OF PEPTIDASES IN *TRYPANOSOMA CRUZI*-HOST CELL

Interaction

Peptidases play a key role in the life cycle of protozoan parasites and are relevant for several aspects of host-parasite interactions [14, 15, 88]. *T. cruzi* peptidases have been implicated in the early events of parasite invasion process, intracellular development and parasite egress [89-91]. Many signaling pathways have been described to promote parasite entry [92-94]. One of these pathways of *T. cruzi* invasion in mammalian cells involves the enzyme Oligopeptidase B (OpbB) [92, 95, 96]. The activity of OpbB induces the release of Ca^{2+} agonist from the parasite that binds to a host cell receptor and activates phospholipase C, leading to IP3-mediated Ca^{2+} release from host cell intracellular stores [97, 98]. The transient increase of cytoplasmic Ca^{2+} levels incites host cell F-actin disassembly at the parasite adhesion site and triggers lysosomal exocytosis, regulated by synaptotagmin VII [99], allowing the formation of the parasitophorous vacuole containing lysosomal markers.

Cruzipain, the major cysteine peptidase in *T. cruzi*, is an alternative mechanism of trypomastigote invasion that also induces Ca^{2+} mobilization and recruitment of lysosomes [23,90,100]. The kinin-mediated pathway is modulated by heparan sulfate glycosaminoglycans, which potently enhances kinin release by *T. cruzi* [38]. Kinin peptide signal is triggered by the B2 type of bradykinin receptor (B2R) in human primary umbilical vein endothelial cells (HUVECs) or Chinese hamster ovary (CHO) cells overexpressing B2R (CHO-B2R) [101]. However, *T. cruzi* invasion is also engaged by B1R with requirement of kininase I [101]. B1R or B2R antagonists and DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) and E-64 inhibitors impair parasite entry [100, 101]. Studies underlying the mechanism of cruzipain proteolytic function have demonstrated that chagasin, member of a family of tight-binding cysteine protease inhibitors, has the ability to bind cruzipain, through its loop domains L2, L4 and L6 and regulate its activity [102, 103]. Besides the involvement of cruzipain on *T. cruzi* invasion [90] and intracellular development [104], this peptidase also plays an important role in parasite egress. Inhibition of cruzipain by the peptidase inhibitor Phe-Ala-FMK reduced *T. cruzi* egress in dose dependent way, achieving a maximal inhibition at 100 μM of inhibitor [89]. In addition, *T. cruzi* presents a homologue of *Leishmania* Gp63, which is GPI-anchored and presents metallopeptidase activity. Blockage of trypomastigote invasion by anti-TcGp63-I antibodies suggests the involvement of this molecule in *T. cruzi* entry [58]. However, the mechanism involved in this process is still unknown.

ULTRASTRUCTURAL STUDIES

Ultrastructural studies in *T. cruzi* have been conducted in order to define the localization and distribution of peptidases in the parasite since the involvement of peptidases in the process of *T. cruzi*-host cell interaction was described [105, 106]. Further studies not only confirmed those findings but also showed the role of peptidases in the intracellular survival, replication and differentiation of the parasite and in disease pathology [23, 32, 58, 90, 107, 108]. Although immunofluorescence techniques have been used to date to locate the peptidases in *T. cruzi*, immunocytochemistry, with the aid of electrondense markers such as colloidal gold particles, is the best choice for enzyme localization due to the reduced dimensions of the parasite. With transmission electron microscopy, it is possible to identify not only the location of the enzymes in the cell but also their sites of accumulation and the compartments in the endocytic/exocytic pathways they flow through (Fig. **1**).

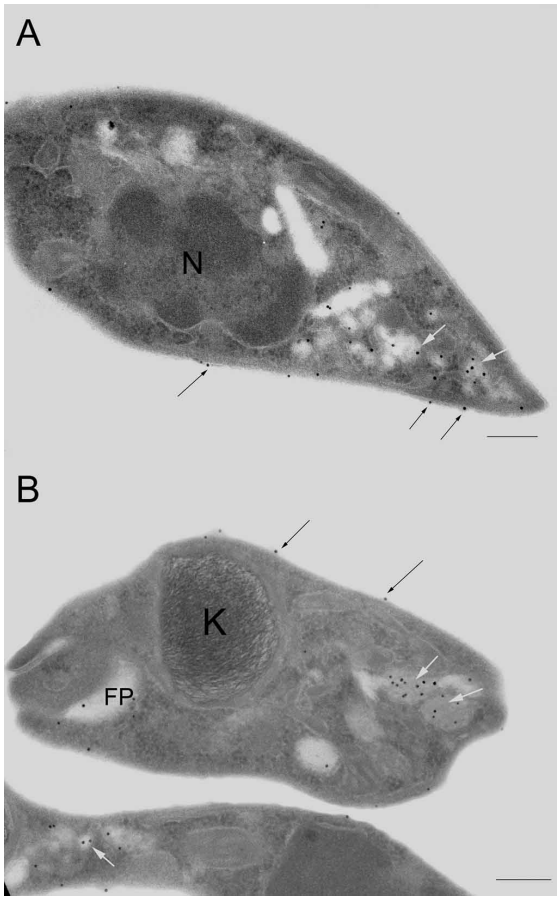


Fig. (1). Immunocytochemical detection of cruzipain in *T. cruzi* amastigotes (**A**) and trypomastigotes (**B**). Parasites were fixed under conditions to preserve antigens and embedded in a hydrophilic resin to allow immunocytochemical detection in ultra-thin sections. Sections were incubated with an anti-cruzipain polyclonal antibody [32] and with goat anti-rabbit IgG coupled to colloidal gold (diameter 8–10 nm). Particles were observed on the surface of both developmental forms (small black arrows), in the flagellar pocket (FP) and in lysosomal-related organelles (white arrows). N, nucleus; K, kinetoplast. Bars= 0.25 μm.

The first peptidase located using immunocytochemical techniques in *T. cruzi* was cruzipain (CZP) [32, 108-110]. Antibodies against CZP were obtained by different groups. Both monospecific polyclonal and monoclonal antibodies against cruzipain-1 or GP57/51 isolated from epimastigotes and its recombinant enzyme named cruzain, as well as a polyclonal antibody against the recombinant cruzipain-2, an isoform that is preferentially expressed by trypomastigotes and amastigotes were produced [111-114].

Immunocytochemical labeling with anti-CZP antibodies revealed the presence of CZP in four main sites: in the cytoplasmic compartments, in the flagellar pocket, on the cell body and on the flagellar membranes (Fig. **1**). The evidence that the cysteine peptidase activity takes place in cytoplasmic compartments came from the studies of Bontempi [115]. Additional immunocytochemical studies showed the enzyme to be located inside enclosed membrane compartments located at the posterior region of the parasite. The positive compartments observed in all developmental forms, are closely related and defined as lysosomal-related organelles (LROs) since they share some functional characteristics with the lysosomes of mammalian cells [34]. In epimastigotes, CZP positive compartments are named reservosomes and have two functions: degradation and storage of macromolecules [116,117]. In trypomastigotes, CZP can be observed in round organelles located between the nucleus and the kinetoplast in a region very close to the flagellar pocket. In amastigotes, CZP is observed in round and/or tubular organelles [34, 108].

The flagellar pocket of all developmental forms of the parasite is frequently labeled suggesting that the enzyme is secreted through this region. Gold particles can be observed associated to the membrane or free inside the pocket. The amount of gold particles within the pocket is variable among the individuals in a sample indicating that the process of CZP release could be modulated by the parasite or related to a particular stage of development since *T. cruzi* samples are naturally not synchronized. The same pattern of labeling is observed in the three developmental forms.

Cruzipain is also observed on the membrane that encloses the cell body and the flagellum but the intensity of labeling is quite different among the developmental forms of *T. cruzi* and varies with the antibody used. Very few or no gold particles indicative of the presence of CZP were seen in association with the surface of most trypomastigote forms [32, 34, 108] labeled with anti-cruzipain-1 antibodies. It is not clear how CZP leaves the Golgi and reaches the reservosomes and the flagellar pocket. However, treatment with drugs shed some light on the different ways CZP could be distributed through the parasite as well as the mechanisms that control the intracellular traffic. The use of cysteine peptidase inhibitors (CPI) resulted in significant alterations in the localization of CZP in epimastigotes, and it seems to be related to the inhibition of the autocatalytic processing of CZP. When CPIs are used there is an accumulation of the enzyme within the Golgi complex that becomes dilated, a decrease in the amount of CZP within reservosomes and a marked increase in the number of vesicles trafficking from the Golgi to the flagellar pocket where a large amount of the inactive enzyme is secreted [118-120].

Local anesthetics, such as dibucaine, also subverted the regular traffic of CZP towards different compartments in epimastigotes. Local anesthetics were found to inhibit the activation of a cytoplasmic, calcium-independent, phospholipase A2 (iPLA2), resulting in a significant inhibition of intracellular, membrane-bound compartment fusions along the endocytic and secretory [121-123]. Dibucaine did not affect the total proteolytic activity in epimastigote forms but induced a delay in the endocytic and exocytic processes. The immunocytochemical detection of cruzipain in Db-treated epimastigotes showed that the amount of CZP in reservosomes displayed a 70% reduction as compared with control cells. In some cells, an accumulation of CZP in the Golgi complex was also observed [124]. More recently, a lysosomal serine carboxypeptidase activity was also described in *T. cruzi* [51]. This enzyme colocates with CZP in the LROs of trypomastigotes and amastigotes [34].

PEPTIDASES AS DRUG TARGET

Although a wide list of known classes of compounds have been assayed *in vitro* and *in vivo* against *T. cruzi*, e.g. allopurinol [125], only two drugs have been approved worldwide for the treatment of Chagas disease, nifurtimox and benznidazole. These drugs were discovered over three decades ago [125-127]. Both of them have significant limitations due to their efficiency solely during the acute phase of the disease. Also, they have significant toxicity and cause unpleasant side effects [128]. The lack of efficient treatment has stimulated efforts to identify new, less toxic and more effective chemotherapy against this parasite [129].

Much of this effort is concentrated on the search for new therapeutic targets for the treatment of Chagas disease. This research uses new technologies such as combinatorial chemistry and structure-based drug design [130].

Peptidases have been widely studied and their inhibitors employed for the treatment of many human diseases, including hypertension and HIV infection and other diseases like diabetes, cancer, thrombosis, osteoporosis, as well as parasitic disease [131].

Peptidase inhibitors have also been considered attractive anti-T.*cruzi* targets. The selective inhibitors of these peptidases have shown the ability to block the proliferation of the parasite and to arrest metacyclogenesis *in vitro* [132].

In *T. cruzi*, the most widely studied peptidase is cruzipain, which is considered an important target of rational drug

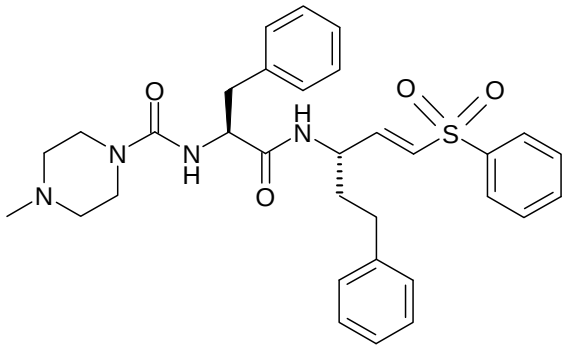
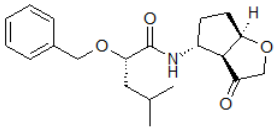
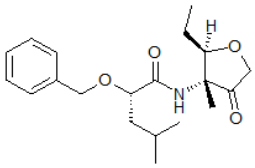
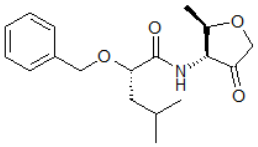
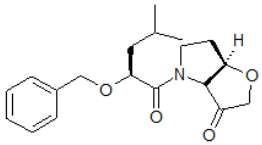
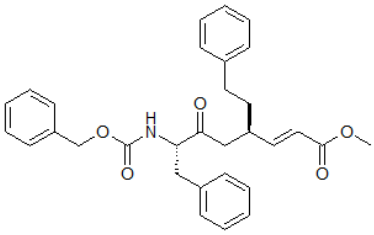
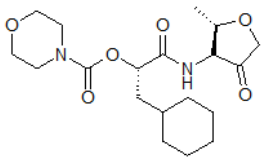
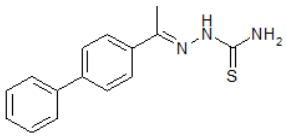
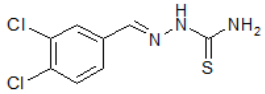
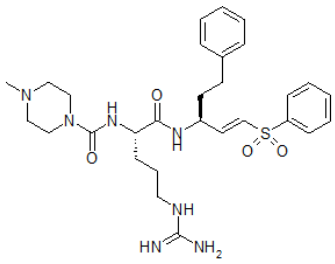


Fig. (2). Chemical structure of the cysteine peptidase inhibitor K777.

Table 2. Patented Cysteine Inhibitors for Chemotherapy of Chagas Disease. Based on Duschak & Couto [134]

Inhibitor structure model (S)	Patent title	Patent number	Inventor
	Inhibitors of cruzipain and other cysteine proteases	WO 02/057246 A2; PCT/GB02/00194	[142]
	Cyclic 2- carbonylaminoketone as inhibitors of cruzipain and other cysteine proteases	US 2004/0127549 A1; PCT/GB02/00190.	[143]
	Inhibitors of cruzipain and other cysteine proteases	WO 02/057248 A2; PCT/GB02/00188	[144]
	Inhibitors of cruzipain and other cysteine proteases	WO 02/05270 A1; PCT/GB02/00184	[145]
	Compounds, Compositions and Methods for treatment of Parasitic Infections	WO 02/48097 A1; PCT/US01/48032	[146]
	Inhibitors of Cruzipain and other Cysteine Proteases	US 2004/0127424 A1. PCT/GB02/00188	[147]
	Anti-parasitic Compounds and Methods of their use	WO 2005/087211; PCT/US2005/007297	[148]
	Thio Semicarbazone and Semicarbazone Inhibitors of Cysteine Proteases and Methods of their use	US 2005/0182121 A1;	[149]
	Proteinase Inhibitors and uses thereof	WO2008/134432; PCT/US2008/061427	[150]

design. This cysteine peptidase is expressed throughout the life cycle and has a wide role in parasite nutrition. Cruzipain has been reported to be involved in the penetration of the parasite into the host cell, as well as in the digestion of immunoglobulins as a defense mechanism. Therefore, the scientific community has demonstrated great interest in the development of new selective cruzipain inhibitors [133]. This target has been explored extensively in recent years as a new and potential chemotherapeutic strategy. It is worth mentioning that 51 patents were issued from 2000 to early 2006 for different drugs targets, both natural and synthetic trypanocidal compounds (ergosterol biosynthesis, synthesis of polyisoprenoids, redox metabolism, DNA nucleotide synthesis, acidocalcisome nucleus, sialic acid transference, cruzipain inhibitors, among others). More than 50% of these patents encompass cruzipain inhibitors (CPI). The CPI chemical structures are diverse and could be divided in synthetic peptidyl and non-peptidyl (thiosemicarbazone) inhibitors. The synthetic peptidyl compounds can be separated into two groups: irreversible (peptidyl diazomethane inhibitors, peptidyl ketone based inhibitors, peptidyl sulphone inhibitors) and reversible (bis-arylacylhydrazides, aryl ureas, ketone based inhibitors, azepanone based inhibitors, nitrile based inhibitors) inhibitors [127,134].

Many of these inhibitors have presented a lack of selectivity, and therefore show toxicity or have shown to be potent CPIs but with little effectiveness against *T. cruzi* in cell culture [114, 135, 136]. Accordingly, a new generation of CPIs has been synthesized with chemical modifications to improve specificity and *in vivo* stability, minimizing toxicity. In this regard, K777 (N-methyl-piperazine-urea-FhF-vinyl-sulfone-phenyl) may be highlighted (Fig. **2**) as a vinyl sulfone that is in late-stage preclinical development, showing good efficacy against different organisms, including *T. cruzi* [127, 136, 137]. In addition, novel CPI classes were studied, presenting the same activity than nifurtimox without its toxicity, for example, quinoxaline-N-acylhydrazone derivatives [138]. Moreover, other peptidases of *T. cruzi* have been identified as promising targets for the design of potential trypanocidal drugs, such as *T. cruzi* prolyl oligopeptidase (Tc80) a serine protease. The synthetic prolylprolyl isoxazoles and prolylprolyl isoxazolines, potent inhibitors of human and trypanosomal prolyl oligopeptidase reduce the invasive capacity of *T. cruzi in vitro* with ED50 in the lower μM range [139, 140]. Although some metallopeptidases and proteasome (threonine peptidases) have been correlated with the *T. cruzi* virulence factor, no studies concerning their inhibitors are available for *T. cruzi* [127].

In a recent review of patented drugs for chemotherapy of Chagas disease, Duschak & Couto [141] described several patented cysteine peptidases inhibitors that could be potentially used for Chagas disease. Table **2** summarizes some of them and new cysteine-inhibitors were added to the list.

PERPECTIVES

Chagas disease affects millions of individuals in the Americas. Since the current drugs present serious side effects and do not cure the chronic infection [128], it is critically important to understand the process of cellular infection at the molecular and structural levels to design novel inhibitors to block *T. cruzi* infection. Peptidases are amazing molecules both because of their diversity in structure and range of biological functions [16]. Studies of peptidases have already revealed exciting new insights into the biology of parasitic protozoa [24]. Future directions in this area must consider analyzing the molecular and cellular basis of *T. cruzi* peptidases that are important for mammalian host infection. The candidate genes coding for *T. cruzi* peptidases and peptidase inhibitors involved in this process need to be intensely studied [92]. The progress made in this area may also facilitate the development of novel cell-based therapies to improve the health conditions of current Chagas disease patients [38].

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