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Metacaspases, Autophagins and Metallocarboxypeptidases: Potential New Targets for Chemotherapy of the Trypanosomiases

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Abstract: During the last decade, *de novo* drug discovery approaches have come into focus due to the increased number of parasite pathogen genomes sequenced and the subsequent availability of genome-scale functional datasets. In order to prioritize target proteins, these approaches consider traits commonly thought to be desirable in a drug target, including essentiality, druggability (whether drug-like molecules are likely to interact with the target), assayability, importance in life-cycle stages of the pathogen relevant to human health, and specificity (i.e. the target is absent from, or substantially different in, the host). Proteases from protozoan parasites have become popular drug targets since these enzymes accomplish both housekeeping tasks common to many eukaryotes as well as functions highly specific to the parasite life style. *Trypanosoma cruzi*, the parasitic flagellate, agent of Chagas Disease, contains several cysteine, serine, threonine and metallo proteinases. This review will deal with peculiar families described in this parasite. Among them, two eukaryote homologues of the carboxypeptidases *Taq* are promising targets due to their particular phylogenetic distribution. Also absent in metazoans, metacaspases are essential peptidases playing important roles in cell growth, death and differentiation of trypanosomatids. Finally, autophagins are involved in the regulation of a conserved degradative pathway, the autophagy pathway, and result important for parasite survival under nutritional stress conditions and differentiation. Although so far there are no specific inhibitors for these families, the increasing knowledge of their biochemical properties, including substrate specificity, crystal structure, and biological functions, is an essential step towards the development of inhibitors.

Keywords: Autophagin, carboxypeptidase, Chagas disease, inhibitor, metacaspase, metallopeptidase, cysteine proteinase, peptidase, *Trypanosoma brucei, Trypanosoma cruzi*.

1. INTRODUCTION

Trypanosomatids are flagellated Protozoa belonging to the Order Kinetoplastida; some of them are pathogens which are the agents of neglected diseases, namely the American trypanosomiasis, Chagas disease, caused by *Trypanosoma cruzi*, the African trypanosomiasis, sleeping sickness, caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, and the different leishmaniases, caused by *Leishmania* spp. (Table **1**). There are no vaccines to prevent these diseases, and, although chemotherapy for them has been available for many years, the drugs used at present are toxic and/or have unpleasant side effects, and/or have only limited efficacy. There is, therefore, an urgent need to develop efficient and less toxic drugs, affordable to the populations in need of them, which are in general poor people living in poor countries [1, 2].

Although most, if not all, the available drugs have been developed empirically, over the last decades the concept of rational chemotherapy has arisen, meaning that the best approach would be to develop inhibitors against suitable targets, namely molecules from the parasites which are essential for them and are either absent in the mammalian host, or have different properties which allow their selective inhibition with a low potential for toxicity in the host. These compounds should have activity *in vitro* against the mammalian stages of the parasites but more importantly *in vivo* in an animal model. It is also favorable that the compounds possess special pharmacokinetic properties such as long terminal half-life and large volumes of distribution. Lastly, since drugs need to be orally administrated, a good oral bioavailability is a desirable property.

Among the possible targets, proteolytic enzymes have been proposed as important candidates, since proteinases from parasites have been shown in a number of cases to be very important, playing roles which go far beyond the digestion of ingested proteins. Host cell invasion and egress, encystation, excystation, catabolism of host proteins, differentiation, cell cycle progression, cytoadherence, and both stimulation and evasion of host immune responses, are some processes in which this group of enzymes is involved [3]. Therefore, the possibility of developing selective inhibitors of key proteinases of pathogenic parasites is currently explored as a novel chemotherapeutic strategy. One example is the major cysteine proteinase from *T. cruzi*, cruzipain (also known as cruzain or GP57/51), which has been shown to be a virulence factor for the parasite and has thus been validated

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Table 1. Diseases Caused by Trypanosomatids and Characteristics of the Parasites

Disease	Parasite	Major Stages in Life Cycle	Vector
Chagas disease	*Trypanosoma cruzi*	Replicative epimastigote in vector.	Triatomine insects
		Intracellular replicative amastigote in the mammal.	
		Non-replicative, infective bloodstream trypomastigote in the mammal.	
Sleeping sickness	*Trypanosoma brucei gambiense* *Trypanosoma brucei rhodesiense*	Replicative long slender trypomastigote in the mammal.	*Glossina* flies
		Non-replicative short stumpy trypomastigote in the mammal.	
		Replicative procyclic trypomastigote in the vector.	
		Replicative epimastigote in the vector.	
		Non-replicative metacyclic trypomastigote in the vector.	
Leishmaniases	*Leishmania* spp.	Replicative promastigote in the vector.	*Phlebotomus* and *Lutzomya* sandflies
		Non-replicative metacyclic promastigote in the vector.	
		Intracellular replicative amastigote in the mammal.	

as a target; indeed, an inhibitor of the enzyme, K11777, is in the last phase of pre-clinical trials at present [4]. More recently an analog of K11777, namely WRR-483, was also shown to be an effective cruzipain inhibitor with trypanocidal activity in cell culture and in a mouse model of acute Chagas' disease [5]. In *T. brucei* a lead inhibitor of cathepsin B, a papain-like peptidase essential for parasite growth (presumably due to its role in iron acquisition via lysosomal degradation of host transferrin), has been recently developed. This compound has potent anti-*Tb*CatB activity, good pharmacokinetic properties, is non-toxic and increased survival time in *T. brucei* infected mice [6].

Trypanosomatids contain, in addition to enzymes belonging to the C1 family, like cruzipain and the homologous enzymes present in *T. brucei* and the leishmanias, other proteolytic activities which either have properties different from their mammalian counterparts, or are just absent in mammals, and are therefore eventual candidates for the development of lead compounds, which may result in suitable drugs for treatment of the diseases. The present review will deal with three enzyme systems which are under investigation by our research group, as well as by other groups: the autophagins, which are essential in the autophagic pathway, which seems to have significant differences with the homologous pathway in mammals; and two enzyme families, the C14 family metacaspases and the M32 family metallocarboxypeptidases, which do not have orthologs in the mammalian genomes completed so far. Some of the literature covered in this review has also been quoted in a recent more comprehensive review on the peptidases of *T. cruzi* [7]. General information about classification and nomenclature of peptidases can be found at MEROPS database (http://merops.sanger.ac.uk) [8].

2. METALLOCARBOXYPEPTIDASES

The M32 family of metallocarboxypeptidases (MCPs) has been extensively characterized in terms of molecular structure. This group of MCPs was initially thought to be restricted to Bacteria and Archaea [9]. *Thermus aquaticus* carboxypeptidase (*Taq*CP), an extremely thermostable enzyme with broad substrate utilization, was the first member of this family to be described [10-12]. Later homologous genes, probably acquired by horizontal gene transfer, were discovered within the Trypanosomatidae family [9]. These organisms, plus some green algae actually constitute the only eukaryotic groups that have M32 genes, making these enzymes attractive potential targets for the development of new drugs against these organisms.

In particular, within trypanosomatids, the M32 family seems to have followed different evolutive pathways. Thus, *T. cruzi* encodes two M32 paralogs with 64% sequence identity, named *Tc*MCP-1 and *Tc*MCP-2. These enzymes differ in substrate specificity and expression throughout the parasite life cycle. *Tc*MCP-1 is expressed in the four main stages of the parasite, whereas *Tc*MCP-2 is a species-specific gene whose expression is restricted to the epimastigote and metacyclic trypomastigote stages, in the insect vector [9]. Substrate utilization of both *T. cruzi* MCPs is opposite and complementary. Thus, *Tc*MCP-2 prefers aliphatic, neutral and aromatic residues at the P1' position (a carboypeptidase A-*like* substrate preference), while *Tc*MCP-1 acts best on basic residues (carboxypeptidase B-*like* substrate utilization) [9]. The related parasite *T. brucei* has reduced to a minimum the M32 protein repertoire. This organism presents a single M32 gene (displaying 72% identity to its ortholog, *Tc*MCP-1). Both MCP-1 enzymes share many characteristics, including P1' preference and pattern of expression, although there are some differences concerning to P1 preference for substrate utilization, which confers unique properties to these hydrolases when acting on synthetic tripeptides [13]. *Leishmania* spp., in the other hand, is characterized by the presence of multiple M32 paralogs. In particular, *Leishmania* spp. conserved a synthenic MCP-1 *like* gene, but expanded to three the number of species-specific genes. One of these species-

specific genes was characterized in *L. major* by Isaza and coworkers. This enzyme, confined to the promastigote stage of the parasite, presented a wider P1' preference for substrate utilization, compared to MCP-1 *like* enzymes [14].

Access to detailed three-dimensional structural information of a protein target can streamline many aspects of drug discovery. In this direction, the crystal structure of *Tc*MCP-1 not only provided a scaffold for rational drug design, but also shed light on the mechanism of M32 substrate recognition. In particular, the overall structure of *Tc*MCP-1 was determined at 2.3 Å resolution [15]. The enzyme is an homodimer, comprised by two identical subunits with its most pronounced feature being a deep substrate binding cleft. The active site lies midway along this cleft and contains the canonical HEXXH motif found in M32 enzymes. This motif includes the active-site glutamic acid residue at position 268, flanked by two histidine residues (positions 267 and 271) that co-ordinate the catalytic metal ion (α10 helix) (Fig. **1**). The overall architecture of *Tc*MCP-1 is not restricted to the M32 family but it is also conserved across diverse families of dipeptidil carboxypeptidases and endopeptidases [15]. Examples are neurolysin (PDB 1i1i, *rsmd* 2.96 Å C α-atoms) and thimet oligopeptidase (PDB 1s4b, *rsmd* 3.26 Å C α-atoms), two enzymes belonging to the family M3. More distantly, though significantly related to *Tc*MCP-1, are human angiotensin-converting enzyme (ACE1, PDB 1uzf, rsmd 3.03 Å C α-atoms) and ACE-like carboxypeptidase (ACE2, PDB 1r4l, *rsmd* 3.01 Å C α-atoms). These enzymes, although presenting low sequence identity (11-13%), retain the general topology, which suggests an evolutionary relationship between them [15, 16]. The high degree of identity between trypanosomatids' MCPs has been used to map critical residues. Homology models of *Tc*MCP-2 based on the crystal structure of *Tc*MCP-1 were utilized to explain the different P1' substrate preference of both enzymes. In this way, and by constructing cross-mutants, it was determined that Met304, located within the *Tc*MCP-1 active site (Arg304 in *Tc*MCP-2), was responsible for carboxypeptidase A/B-type substrate discrimination [15]. A similar approach was used to delimit the S1 subsite of *Tb*MCP-1. In this case, Ala414 was found to play a major role limiting the activity of *T. brucei* enzyme over the Abz-FVK(Dnp)-OH substrate [13]. Arg348, a conserved amino acid residue, proposed to form a salt bridge with the C-terminal carboxylate group of substrate, was essential since its replacement by Ala abolished the MCPs' activity [13]. Moreover, Arg348 is structurally conserved in other monocarboxypeptidases of the clan MA, namely, ACE2. These data plus data on 3D structure and substrate specificity constitute a valuable tool in order to design inhibitors that eventually would allow to explore the M32 family biology and enzymology.

The biological function of the M32 peptidases is still unknown, but it has been suggested that the trypanosomal enzymes might play a role in peptide catabolism. In particular, Isaza and co-workers suggested that a species-specific M32 MCP found in *L. major* could facilitate the growth of the promastigote form of the parasite participating in the degradation of peptides and proteins to free amino acids [14]. Other possible functions came from the structural similarity of this family with mammalian metallopeptidases, including ACE and ACE2. Both enzymes are zinc peptidases that play a central role in the renin–angiotensin system. Taking into account the marked substrate preference reported for some M32 MCPs a role in the regulation of the metabolism of small peptides could not be excluded [13]. In this direction, it has been shown that *Tc*MCP-1 could produce exclusively des-Arg bradykinin (BK) [13], which has been reported to facilitate *T. cruzi* cell invasion through B1 receptors since addition of [Leu8]-des-Arg9-BK, a B1 receptor antagonist, is known to reduce the parasite infectivity [17]. Recently, it has been suggested that these peptidases are secreted by Trypanosomatids, thus making feasible this hypothesis [18, 19].

Although to date there are not specific inhibitors of the M32 family, selective inhibitors of metallocarboxypeptidases with carboxypeptidase B-like specificity including GEMSA or Plummer's inhibitor were informed to be effective on trypanosomatids MCP-1 enzymes [13]. Moreover Plummer's inhibitor was found to selectively decrease *T. cruzi* infectivity for B1R-expressing cells [17]. Other compounds, including DX600, a 26-amino-acid residues N-terminal acetylated and C-terminal amidated peptide, selected to specifically inhibit ACE2 [20], also presented a modest inhibition on *T. brucei* and *T. cruzi* enzymes [13]. Thus, for trypanosomatid MCPs, not only essential residues involved in substrate binding, such as Arg348 are conserved compared with ACE2, but also the response to some inhibitors [13]. These facts open up the possibility to test on the trypanosome MCPs other inhibitors already developed, or to be developed in the future, for ACE2.

3. METACASPASES

Metacaspases were first identified as distant relatives of animal caspases based on certain degree of sequence similarity (including the conservation of the Cys-His catalytic dyad) and a predicted common secondary structure [21]. Since metacaspases were precisely found in the genomes of plants, fungi and protozoa, where cell death with apoptotic features had been largely documented although they do not possess classical caspases, this novel family of peptidases was at the beginning postulated as interesting candidates for caspases orthologues. However, it was later shown that metacaspases possess a completely different substrate specificity compared to that of caspases. While caspases cleave peptides and proteins after aspartic acid residues, all metacaspases studied so far have specificity towards basic (Arg/Lys) amino acids. Another important difference among caspases and metacaspases is that the latter are absolutely dependent on calcium for activity, a property that has never been described for a caspase to date [22-28]. Metacaspases may be classified into two major groups: type I metacaspases present N-terminal extensions containing protein-protein interaction motifs preceding the catalytic domain, whereas type II metacaspases present short N-terminal regions but insertions inside the catalytic domain specifically dividing the regions homologous to caspase fragments p20 and p10. Type I metacaspases are widely distributed in non-metazoan organisms but type II metacaspases are only present in plants. The most striking difference among both types of metacaspases is that type I metacaspases seem not to require any processing to display activity while type II enzymes need a self proteolytic cleavage in the p20-p10 spacer region in order to be active [22, 25, 26, 28].

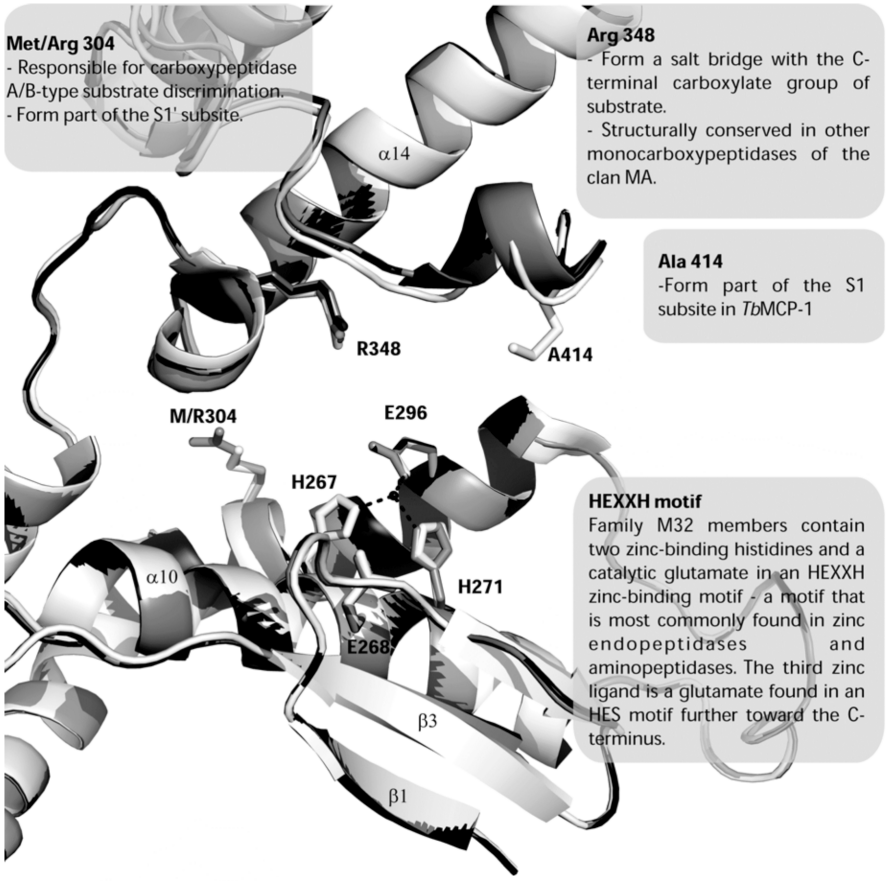

Fig. (1). Structure of *T. cruzi* and *T. brucei* metallocarboxypeptidases. Homology models of *Tb*MCP-1 (black) and *Tc*MCP-2 (grey) based on the crystal structure of *Tc*MCP-1 (white, pdb id 3dwc) were utilized to map the positions of the potentially critical residues involved in substrate specificity. The figure shows a close-up view of protozoan MCP active-site cleft. Residues relevant for catalysis as well as residues that differ between enzymes are shown as sticks. Figures were prepared using the program PyMOL. (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC).

It seems likely that metacaspases could represent versatile peptidases modulating cell death pathways or other non-related biological processes [29]. The first experimental evidence pointing towards a role for a metacaspase in a programmed cell death pathway was obtained in yeast [30]. Apoptosis induced by hydrogen peroxide was abrogated after disruption and stimulated after overexpression of the yeast metacaspase; and yeast metacaspase was shown to mediate the death process in aged cultures. Since then, several authors have reported metacaspase-dependent or independent cell death pathways. More recently, multiple roles for the yeast metacaspase were proposed including control of the cell cycle progression and clearance of protein aggregates among others [31, 32].

In plants, the relationship between metacaspases and apoptosis seems to be less uncertain. It has been shown that plant metacaspases can modulate cell death during embryogenesis, under oxidative stress or as part of the hypersensitive response [33, 34]; however, the molecular mechanisms underlying it are still far from being clear and depend on the identification of metacaspase natural substrates. At present, TSN (Tudor staphylococcal nuclease) is the only protein shown to be cleaved by a metacaspase *in vivo*, during both developmental and stress-induced cell death. TSN knockdown leads to activation of ectopic cell death during reproduction, impairing plant fertility [35].

The possibility that metacaspases are involved in a cell death pathway makes them an attractive research field in the medically relevant trypanosomatids *L. major*, *T. brucei* and *T. cruzi*. All three trypanosomatids possess a single-copy metacaspase gene that has a C-terminal extension rich in Pro, Gln and Tyr, in addition to the catalytic domain. These proteins were named *Lmj*MCA, *Tb*MCA5 and *Tc*MCA5 in *L. major*, *T. brucei* and *T. cruzi*, respectively. *T. brucei* and *T. cruzi* (but not *L. major*) also bear multiple copy genes encoding the catalytic domain alone lacking the C-terminal region (4 genes in *T. brucei* named *Tb*MCA1-4 two of which are predicted to encode inactive peptidases due to mutations in the catalytic dyad, and about 16 genes in *T. cruzi* called *TcMCA*3) [24, 36, 37]. The biochemical properties of all trypanosomatid metacaspases are consistent with the features first reported for plant metacaspases, namely the specificity for basic amino acid residues at P1 and the requirement of calcium for activity. The only metacaspase from *L. major* has been reported to undergo self-proteolytic processing that removes the C-terminal extension and enhances its peptidase

activity *in vitro*. In contrast, *Tb*MCA2-3 as well as *Tc*MCA3 and *Tc*MCA5, do not require any processing in order to display enzymatic activity [24-26].

The role of these enzymes in trypanosomatids is still elusive. It has been demonstrated that overexpression of *Tc*MCA5 in *T. cruzi* renders epimastigotes more susceptible to programmed cell death induced by fresh human serum [37] and that inducible overexpression of *Tc*MCA5 lacking the C-terminal extension in epimastigotes leads to cell death with apoptotic features [26]. In good agreement with these results, the orthologous protein from *L. major* was able to replace yeast metacaspase for the induction of cell death during ageing and the catalytic domain alone is sufficient to enhance sensitivity of parasites to hydrogen peroxide [24]. Conversely, in *T. brucei* bloodstream forms, triple null mutants (*Δmca2/3 Δmca5*) did not impair cell death induced by prostaglandin D_2 when compared to wild type parasites. Indeed, it has been shown by triple RNAi analysis that *T. brucei* metacaspases are essential for normal cell cycle progression of the bloodstream form, with parasites accumulating in pre-cytokinesis [38]. *L. major* metacaspase seems also to be essential as it was not possible to generate a null mutant and evidence from overexpression of *Lmj*MCA in promastigotes also supports a role in cell cycle control [39]. More recently, a tight regulation of *Tc*MCA3 levels in *T. cruzi* was proposed to be crucial for the normal completion of the cell cycle in the replicative stages epimastigote and amastigote (Fig. **2**). Furthermore, and acting antagonically to *Tc*MCA5, *Tc*MCA3 has been shown to protect epimastigotes from naturally occurring cell death, and to stimulate differentiation to infective metacyclic trypomastigotes [26]. All together, these results suggest that metacaspases could play important roles in the life cycle of trypanosomatids and brings to light the close relationship between cell division, death and differentiation in these ancient unicellular eukaryotes.

Regardless of metacaspases being involved in cell death related or unrelated pathways, it appears evident that they play key functions. This, together with the fact that they are absent in humans and display low sequence similarity with caspases, makes them very attractive potential targets for chemotherapy. Recently, based on substrate specificity, a first series of inhibitors acting at a low micromolar range has been developed for *Tb*MCA2 that could constitute the basis for a rational drug design [40]. The compounds inhibit *Tb*MCA2, possess modest antiparasitic activity, but have excellent selectivity when compared to mammalian caspases. The best *Tb*MCA2 inhibitor, a benzothiazole derivative of a Cbz-blocked L-arginine at the C-terminus, had a IC50 value of 0.6 μM, and was able to inhibit the growth of *T. brucei* bloodstream trypomastigotes and *T. cruzi* intracellular amastigotes with IC50 values of 32.9 and 20.9 μM, respectively. Optimization efforts are ongoing.

Very recently, the first three-dimensional structure of a metacaspase (*Tb*MCA2) was determined at 1.4-Å resolution [41]. The structure consists of a core caspase fold, but with an unusual eight-stranded β-sheet that stabilizes the protein in a monomeric form. The absence of structurally conserved self-cleavage sites and dimerization interfaces in *Tb*MCA2 is consistent with the previous observation that type I metacaspases have a mode of activation different from that of the caspases. The presence of essential aspartic acid residues in the predicted S1 binding pocket could explain the arginine-specific substrate specificity. The most remarkable feature of *Tb*MCA2 structure is that the N terminus surrounds the protein, goes across the catalytic dyad and regulates substrate access. Calcium binding to four conserved aspartic acid residues apparently induces a conformational change stabilizing the active site and regulating activity. This knowledge provides a means towards designing specific inhibitors of metacaspases that can potentially be used for the development of novel drugs against parasitic diseases.

4. AUTOPHAGINS

The autophagy pathway is a conserved mechanism present in all eukaryotic cells to degrade and recycle macromolecules and even entire organelles. The target is isolated by double membrane vesicles called autophagosomes which fuse with the lysosomes promoting the degradation of the enclosed material [42]. Genetic screens performed in yeasts allowed the identification of most of the genes responsible for autophagy, resulting in more than 20 autophagy related genes (*ATG*s) and among them, two ubiquitin-like conjugation systems that play essential roles in autophagosome biogenesis [43]. The Atg8 conjugation system acts on vesicle expansion and completion [44]. Atg8 is synthesized in the cytosol as a precursor that requires a defined proteolytic processing, performed by the cysteine peptidase Atg4 or autophagin, near the C-terminus to expose a conserved Gly residue. The exposed Gly on Atg8 is then activated by the E1 enzyme Atg7, transferred to the E2 enzyme Atg3 and finally conjugated to the amino group of phosphatidylethanolamine (PE) which allows a tight association of Atg8-PE with membranes [45, 46]. Atg8 is deconjugated from PE in the outer membrane of the autophagosomes, before fusion with the lysosomes, by the same protease Atg4, and released to the cytosol in order to be reused for new vesicle formation. In the Atg12 conjugation system, the ubiquitin-like protein Atg12 is covalently bound via its C-terminal glycine residue to an internal lysine residue present in Atg5 by the action of the E1- and E2-like enzymes Atg7 and Atg10 [47, 48]. The Atg12-Atg5 associates with Atg16 and then binds to the outer membrane of the nascent autophagosome. The Atg5-Atg12-Atg16 complex is supposed to drive the deformation of the autophagosomal membrane and dissociates from it before completion (Fig. **3**).

Bioinformatic analysis of the genome of *T. cruzi* revealed the presence of all the components of the Atg8 conjugation system, with two putative Atg4 and Atg8 proteins [49]. Yeast complementation studies support a conserved role for both *T. cruzi* Atg4 genes. Moreover, when using the *T. cruzi* purified recombinant proteins, N-terminal sequencing of the fragments of the Atg8 proteins released by the Atg4 peptidases showed that in all cases they were processed after the conserved Gly residue. However, only one of the Atg8 sequences was able to complement an Atg8 yeast deletion mutant. Consistent with this finding, immunofluorescence studies showed that only this variant localized in autophagosome-like vesicles under starvation conditions both when a HA-tagged version of the protein was overexpressed in epimastigotes and anti-HA monoclonal antibodies were

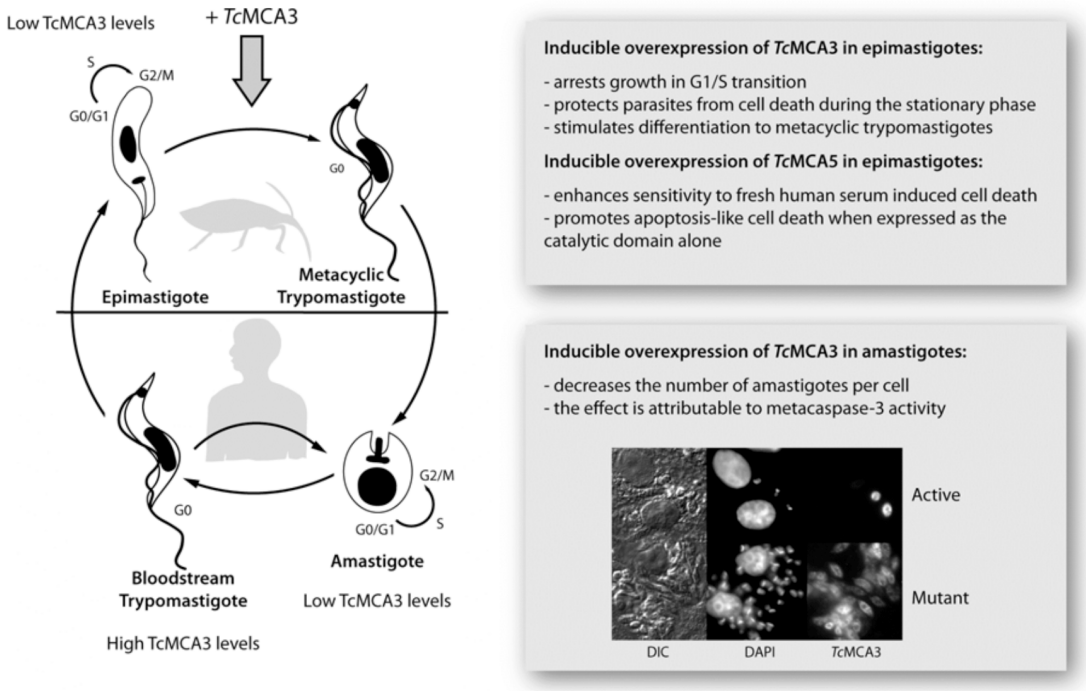

Fig. (2). Timely-regulated expression of metacaspase-3. In the insect vector active metacaspase-3 levels need to be tightly regulated, being low in the replicative stage, the epimastigote; and higher at the non-replicative mammalian infective form, the metacyclic trypomastigote. Overexpression of active metacaspase-3 in epimastigotes during exponential growth phase is detrimental, leading to DNA synthesis blockage. However, when overexpression is achieved during the stationary phase of the culture, parasites are protected from programmed cell death and differentiation to metacyclic trypomastigotes is stimulated. When active metacaspase-3 is overexpressed in intracellular amastigotes, the number of parasites per cell is decreased suggesting again that replicative stages should possess lower amounts of this enzyme. Immunofluorescence studies of Vero cells infected with amastigotes shows the drastic reduction in the number of parasites per cell in the latter case. DIC, differential interference contrast microscopy; DAPI stain the nuclei of Vero cells as well as the nuclei and kinetoplast of amastigotes and *Tc*MCA3 active and mutant (inactive) forms were overexpressed as Flag fusion proteins and visualized with anti-Flag antibodies. This shows that the enzyme activity is required for the effect.

used or when wild-type parasites were analyzed with polyclonal antibodies specific to this protein [49]. It seems likely that the other Atg8 variant could actually represent a singular version of Atg12 requiring cleavage prior to conjugation to Atg5. In fact, it was very recently shown that this could be the case for the orthologous gene from *L. major* [50, 51].

The activation of the autophagy pathway during starvation is physiologically relevant as it occurs naturally to the epimastigotes in the gut of the insect vector, which suffers long periods of lack of food (up to 12 months) [52]. Moreover, starvation has been suggested to represent a signal for metacyclogenesis, and an essential role for autophagy in the differentiation of protozoan parasites is emerging [53, 54].

Cruzipain is the major digestive peptidase present in the lysosomal-like vesicles of epimastigotes, the reservosomes, and it is reasonable to speculate that it could be involved in the last phase of autophagy, the degradation of autophagosomal contents. It is noteworthy that cruzipain overexpression enhances metacyclogenesis [55], its inhibition inhibits this differentiation step [56], and also hampers the growth and differentiation of the parasite inside the mammalian cell [57]. It is quite possible; therefore, that both, autophagy and cruzipain, are involved, together with the proteasome, in the differentiation events taking place inside the mammal.

There are human pathological conditions where autophagy is believed to be overly active, including neurodegeneration and cancer. Therefore, targeting this pathway has been recently suggested as a new strategy for treatment of these diseases. However, chemical inhibitors are scarce and used only for research purposes. The most widely used inhibitor, 3-methyladenine, requires millimolar concentrations to inhibit class III phosphatidilinositol-3-kinases. Bafilomycin A1 inhibits the vacuolar type H^+-ATPase blocking acidification of lysosomes and endosomes but when used for longer periods it starts to affect other cellular processes. One possibility to inhibit specifically the autophagy pathway would be to interfere with Atg8 processing and deconjugation using small synthetic inhibitors of autophagins. A pilot high-throughput screen for human autophagin-4B inhibitors has been recently reported using two small collections of compounds enriched in bioactive molecules [58]. Four inhibitors were detected, with IC50 values ranging from 1 to 3 μM. Considering the evolutionary conservation of the substrate specificity of this proteinase, it is possible that these inhibitors could also be tested, perhaps as a combined therapy with cruzipain inhibitors, for the treatment of Chagas disease.

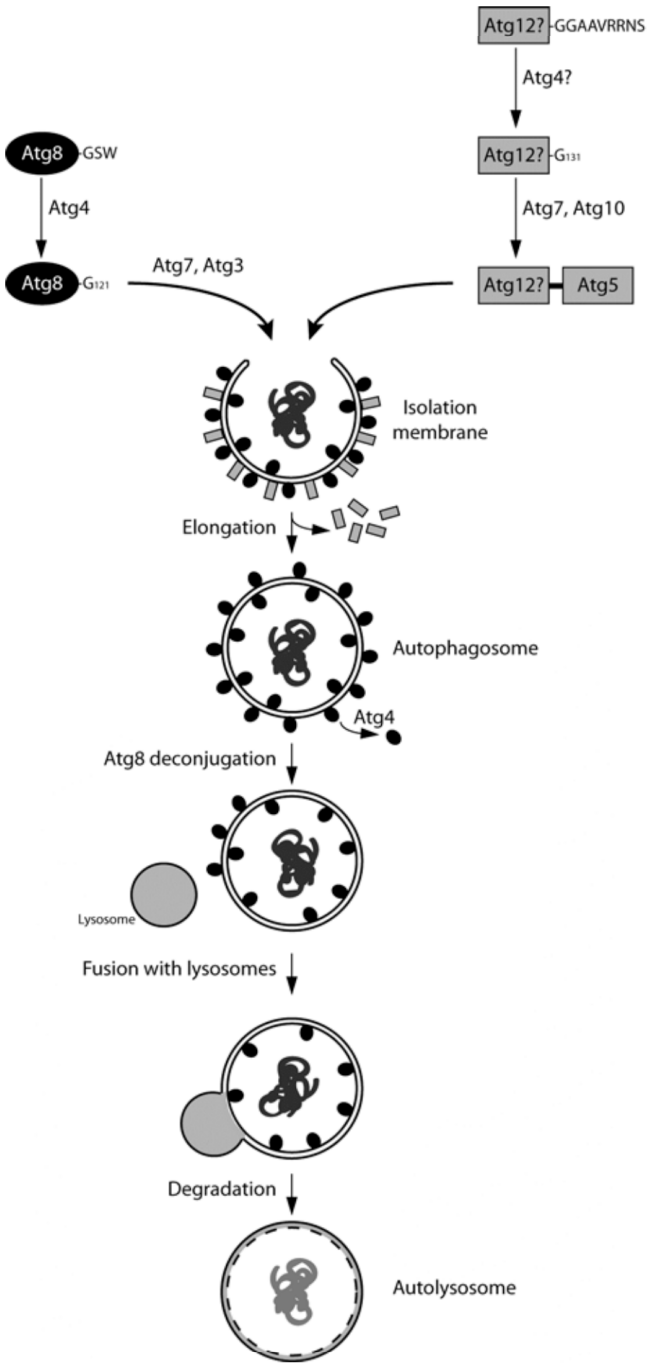

Fig. (3). Molecular mechanisms of autophagosome formation. In eukaryotic cells, two ubiquitin-like conjugation systems are involved in autophagosome biogenesis. Trypanosomatids contain a canonical Atg8 conjugation system but seem to possess an unusual Atg12 conjugation pathway. In the Atg8 classical system, the ubiquitin-like protein Atg8 is synthesized as a precursor which needs to be processed by a cysteine peptidase named autophagin or Atg4. Processed Atg8 exposes a Gly residue at the C-terminus and it is first activated by the activating (E1) enzyme Atg7, then transferred to the conjugating enzyme (E2) Atg3 which in turns promotes its covalent binding to phosphatidyethanolamine (PE) allowing a tight association between Atg8 and the autophagosomal membranes. The most remarkable feature of the Atg12 system is that, at variance with all other ubiquitin-like Atg12 proteins, trypanosomatid Atg12 seems also to be produced, as Atg8, as a precursor form that is likely to be processed in an analogous fashion by Atg4. Processed Atg12 is conjugated to Atg5 in a process mediated by Atg7 (the E1 enzyme common to both pathways) and Atg10 (an specific E2 enzyme). The Atg12-Atg5 conjugate forms a transient coat that drives the deformation of the autophagosomal membrane during vesicle formation. Atg8-PE acts on vesicle expansion and completion. Before fusion with the lysosome, Atg8 is deconjugated from PE in the cytosolic face of the autophagosome by Atg4 and is released in order to be reused for new vesicle formation.

5. CONCLUSION

The enzymes reviewed here show a clear potential to be considered as possible new targets for chemotherapy. The M32 metallocarboxypeptidases are perhaps the most outstanding, due to their restricted phylogenetic distribution, but it is still necessary to validate their essentiality for the parasites, using reverse genetics techniques in *T. cruzi* or *T. brucei*; experiments with this purpose are ongoing at present in our laboratory. In the case of the metacaspases and the autophagins, it is clear that these enzymes play fundamental roles in the parasite biology, regulating the progression of the cell cycle in the case of the metacaspases, or participating in the differentiation of parasite stages in the case of the autophagins. In these cases the most important challenge consists in the identification of selective inhibitors for the parasite´s enzymes compared with those in the mammalian, using the libraries of compounds already available, or designed on the basis of our knowledge of the biochemistry and the 3D structure of these enzymes. In the case of metacaspases other workers have developed a first series of inhibitors of the purified recombinant enzyme, which present antiparasitary action and are not toxic to human fibroblasts. In the case of the autophagins, selective inhibitors are being developed, but these have not yet been tested on parasite cultures, and this will be done in the near future.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

ACE1	=	Angiotensin-converting Enzyme
ACE2	=	Angiotensin-converting Enzyme-like Carboxypeptidase
ATG	=	Autophagy-Related Gene
BK	=	Bradykinin
HA	=	Hemmaglutinin

MCA	=	Metacaspase
MCP	=	Metallocarboxypeptidase
PE	=	Phosphatidylethanolamine
TbCatB	=	*Trypanosoma brucei* Cathepsin B
*Tc*MCP-1	=	*Trypanosoma cruzi* Metallocarboxypeptidase-1
*Tc*MCP-2	=	*Trypanosoma cruzi* Metallocarboxypeptidase-2
TSN	=	Tudor Staphylococcal Nuclease

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