Calpains: Potential Targets for Alternative Chemotherapeutic Intervention Against Human Pathogenic Trypanosomatids

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Abstract: The treatment for both leishmaniasis and trypanosomiasis, which are severe human infections caused by trypanosomatids belonging to *Leishmania* and *Trypanosoma* genera, respectively, is extremely limited because of concerns of toxicity and efficacy with the available anti-protozoan drugs, as well as the emergence of drug resistance. Consequently, the urgency for the discovery of new trypanosomatid targets and novel bioactive compounds is particularly necessary. In this context, the investigation of changes in parasite gene expression between drug resistant/sensitive strains and in the up-regulation of virulence-related genes in infective forms has brought to the fore the involvement of calpain-like proteins in several crucial pathophysiological processes performed by trypanosomatids. These studies were encouraged by the publication of the complete genome sequences of three human pathogenic trypanosomatids, *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania major*, which allowed *in silico* analyses that in turn directed the identification of numerous genes with interesting chemotherapeutic characteristics, including a large family of calpain-related proteins, in which to date 23 genes were assigned as calpains in *T. brucei*, 40 in *T. cruzi* and 33 in *L. braziliensis*. In the present review, we intend to add to these biochemical/biological reports the investigations performed upon the inhibitory capability of calpain inhibitors against human pathogenic trypanosomatids.

Keywords: Alternative chemotherapy, calpain, *Leishmania*, monoxenous trypanosomatids, peptidase inhibitor, *Trypanosoma*, virulence.

1. TRYPANOSOMIASIS: A WORLDWIDE PROBLEM OF PUBLIC HEALTH

The Trypanosomatidae family, which belongs to the Kinetoplastida order, is composed of a diverse group of exclusively parasitic protozoa. Trypanosomatids are distinguishable from other protozoa by distinctive organizational features such as the presence of (i) a single flagellum, (ii) a subpellicle microtubule cytoskeleton, (iii) a kinetoplast containing densely packed DNA (kDNA) that is organized in mini- and maxi-circles and that corresponds to the parasite mitochondrial genome, localized near the basal body of the flagellum, (iv) glycosomes, which are membrane-enclosed organelles that compartmentalize the glycolytic enzymes, (v) acidocalcisomes, which are acidic organelles containing calcium and a high concentration of phosphorus in the form of pyrophosphate and polyphosphate that are responsible for calcium homeostasis, maintenance of intracellular pH and osmoregulation, and (vi) flagellar pocket, a specialized region used by trypanosomatids for molecular traffic into and out of the cell (Fig. **1**) [1, 2]. In addition, these microorganisms exhibit unusual molecular phenomena such as antigenic variation, *trans*-splicing, RNA editing and peculiar nuclear organization. The trypanosomatids seem to be able to adapt with ease their energy metabolism to the availability of substrates and oxygen, and this may give them the ability to institute new life cycles if host behavior patterns allow [1, 2].

A fraction of the trypanosomatids has adapted to parasitize humans, such as *Trypanosoma brucei*, the etiologic agent of African sleeping sickness; *Trypanosoma cruzi,* the causative agent of Chagas' disease or American trypanosomiasis; and different species of the genus *Leishmania*, responsible for a wide spectrum of clinical manifestations varying from cutaneous and mucocutaneous to visceral leishmaniasis [3-5]. Despite the great advances in combating infectious diseases over the past century, these parasites continue to inflict a tremendous social and economic burden on human societies, particularly in the developing world. In this sense, there is a general lack of effective and inexpensive chemotherapeutic agents for treating protozoan diseases. Current therapy is limited to a handful of drugs that suffer from unacceptable toxicity, difficulties of administration and increasing treatment failures, since resistance to these

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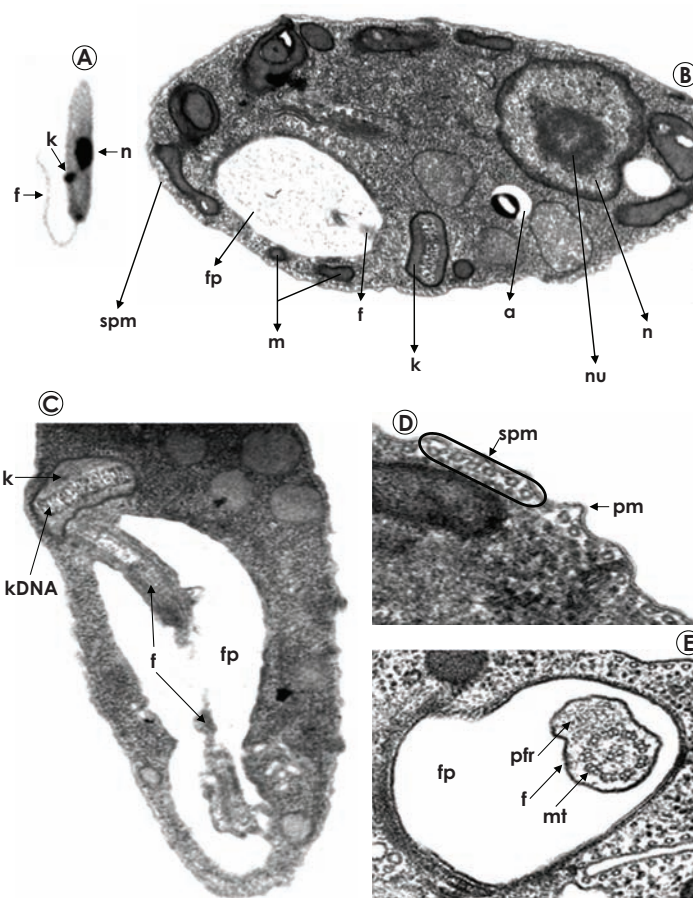Fax: +55-21-2560-8344; E-mail: mbranquinha@micro.ufrj.br

Fig. (1). Representative morphology of a trypanosomatid cell. (**A**) Optical microscopy evidencing the Giemsa-stained parasite. This staining shows the position of the nucleus (n) in relation to the kinetoplast (k) in trypanosomatids. Note the presence of only one flagellum (f). (**B**) Transmission electron microscopy showing the organelles/structures of a trypanosomatid cell. Note in (**C-E**) a higher magnitude of the typical cellular structures. (kDNA) kinetoplast DNA, (fp) flagellar pocket, (spm) subpelicular microtubules along all extension of the (pm) plasmatic membrane, (mt) microtubules and (pfr) paraflagellar region that constitute the flagellum of trypanosomatids, (m) mitochondrion, (a) acidocalcisome and (nu) nucleolus.

compounds has become a severe problem [6-10]. In view of this scenario, the development of new drugs is an urgent need, which has led to the performance of several tests utilizing compounds chosen empirically, or through studies that identify metabolic targets in the parasite. In addition, the variable protein expression by the different trypanosomatid life cycle stages that alternate between vertebrate and invertebrate hosts as well as by distinct strains needs to be taken into account when new therapeutic targets are proposed.

2. PEPTIDASES OF TRYPANOSOMATIDS: POTENTIAL TARGETS FOR DRUG DEVELOPMENT

Peptidases of human protozoan pathogens, specifically in trypanosomatids, have attracted the attention of many laboratories because these enzymes are not only involved in "housekeeping" tasks common to many eukaryotes as well as due to their many roles in highly specific functions to the parasites' life cycles, including pathogenesis [11-16]. Consequently, the study of these hydrolytic enzymes has led to the design of novel proteolytic inhibitors against these pathogens, and the simultaneous evaluation of the anti-trypanosomatid activity of molecules originally developed

for distinct targets and/or cell models [8, 15]. For instance, K777, a vinyl sulfone peptidase inhibitor of cruzipain, the major cysteine peptidase from *T. cruzi,* was effective in curing or alleviating the parasitic infection in preclinical proof-of-concept studies and has now entered formal preclinical drug development investigations [8, 17].

With the publication of the complete genome sequences of *T. brucei, T. cruzi* and *Leishmania major,* which led to the discovery of a large family of calpain-like proteins (CALPs) in the Trypanosomatidae family [18], the possibility of a new therapeutic target has been found.

3. CALPAINS AND CALPS

Calpains are calcium-dependent cysteine peptidases that have been extensively studied in mammalians and that exist in two major isoforms, μ-calpain (calpain 1) and m-calpain (calpain 2), which require micromolar and millimolar concentrations of Ca^{2+}, respectively, for their activation [19]. They are heterodimeric proteins, consisting of a large subunit of approximately 80-kDa and a small subunit of 28-kDa. The large subunit is divided into four domains, where domain I is the N-terminal or prodomain, of unknown function, and do-

main II corresponds to the catalytic core of the enzyme, characterized by the presence of the conserved catalytic triad containing the amino acids cysteine, histidine and asparagine as well as two calcium-binding sites. Domain III is a linker between domain II and domain IV, the latter containing five functional calcium-binding EF-hand motifs that are essential for enzymatic activity and for the dimerization with the small subunit [20]. In addition to these four-domain calpains, proteins referred to as CALPs have been identified in mammals but mainly in invertebrates and in lower eukaryotes, such as fungi, protists, nematodes, plants and invertebrates such as *Drosophila melanogaster* [19, 21-23]. Some of these calpain homologues may differ in amino acid composition within the catalytic triad at domain II, which raised uncertainties as to whether any of these calpain homologues has proteolytic activity [19]. Other calpain homologues lack domain III and/or domain IV, and some contain other types of modules [24], but since the catalytic triad in domain II is intact they may display proteolytic activity [25]. Like mammalian calpains, these homologues are involved in a variety of calcium-regulated cellular processes, such as signal transduction, cytoskeleton remodeling, cellular proliferation and differentiation, sex determination, membrane fusion, environmentally-regulated processes and apoptosis (Fig. **2**) [19, 25].

Calpain activation in humans seems increased during normal aging and in muscular dystrophy, neurological disorders like Alzheimer's, Huntington's and Parkinson's diseases and multiple sclerosis, cataract, arthritis, cancer, strokes, diabetes and in acute traumas including traumatic brain and spinal cord injury and cerebral and cardiac ischemia (Fig. **2**)

[26-32]. The use of calpain inhibitors is demonstrably beneficial in animal models of these diseases; hence, this class of proteolytic enzyme has been proposed as a potential drug target. In this context, the potential clinical utility of these inhibitors has been shown to treat Alzheimer's disease, Duchenne muscular dystrophy and to minimize neuronal death after ischemia [26-32].

Calpain inhibitors are known to block peptidases with varying specificity. *In vitro*, calpain activity may be inhibited by the general cysteine peptidase inhibitors, such as *trans*-epoxysuccinyl-L-leucylamido-4-guanidino-butane (E-64) and leupeptin (Fig. **3**), although their broad specificity and poor membrane permeability render them inadequate for rapid and selective inhibition of calpain in intact cells. More specifically, calpains may be inhibited with synthetic reversible peptide calpain inhibitors, such as Cbz-Val-Phe-H, also known as MDL28170 or calpain inhibitor III (Fig. **3**), which displays high membrane permeability [33]. The majority of calpain inhibitors developed to date are peptide analogues, which can be sub-divided in peptidyl epoxides, peptidyl aldehydes and peptidyl α-ketoamides (Fig. **3**). A major limitation to the therapeutic potential of most of the peptide-based calpain inhibitors is their lack of selectivity for calpains relative to other cysteine peptidases such as cathepsin B and other calcium-activated peptidases [26, 32, 34]. To overcome this approach, there are now many non-peptide calpain inhibitors (Fig. **3**) under development that interact with allosteric sites of calpains and hence demonstrate significant selectivity; these molecules may provide more therapeutic benefit than peptide inhibitors against a variety of human diseases [31].
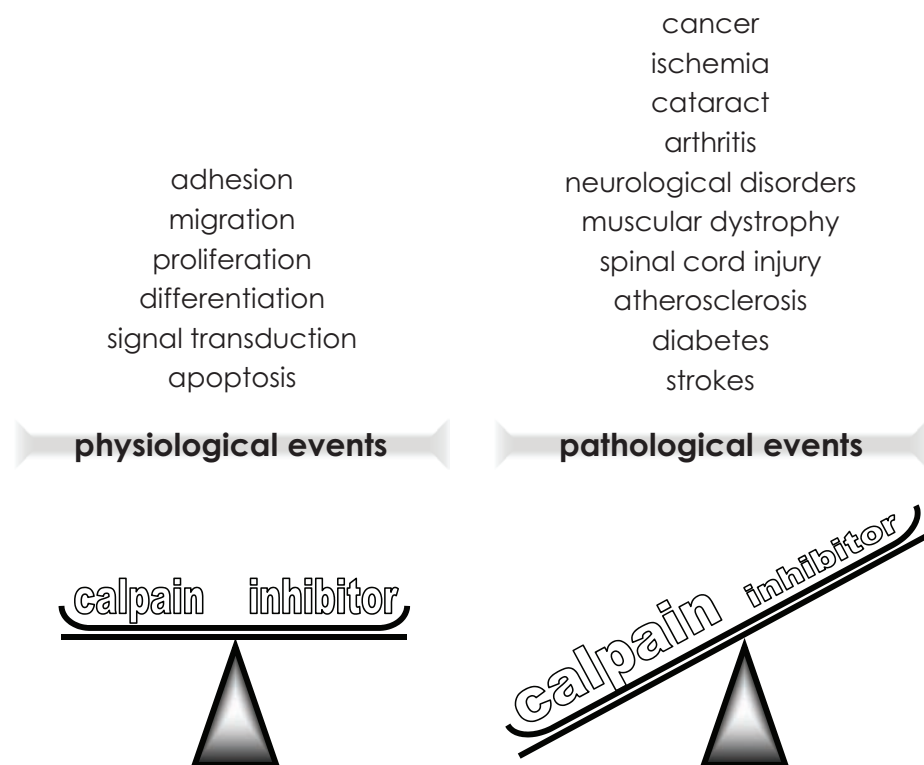
cancer
ischemia
cataract
arthritis
neurological disorders
muscular dystrophy
spinal cord injury
atherosclerosis
diabetes
strokes

adhesion
migration
proliferation
differentiation
signal transduction
apoptosis

physiological events

pathological events

Fig. (2). Physiological and pathological events in which calpain molecules have effective participation in humans. The equilibrium between calpain activity and its natural inhibitor promotes crucial events in order to maintain the homeostasis of a eukaryotic cell. On the contrary, when the calpain expression is exacerbated in relation to its inhibitor, several disorders can be triggered. See [79] for more details.
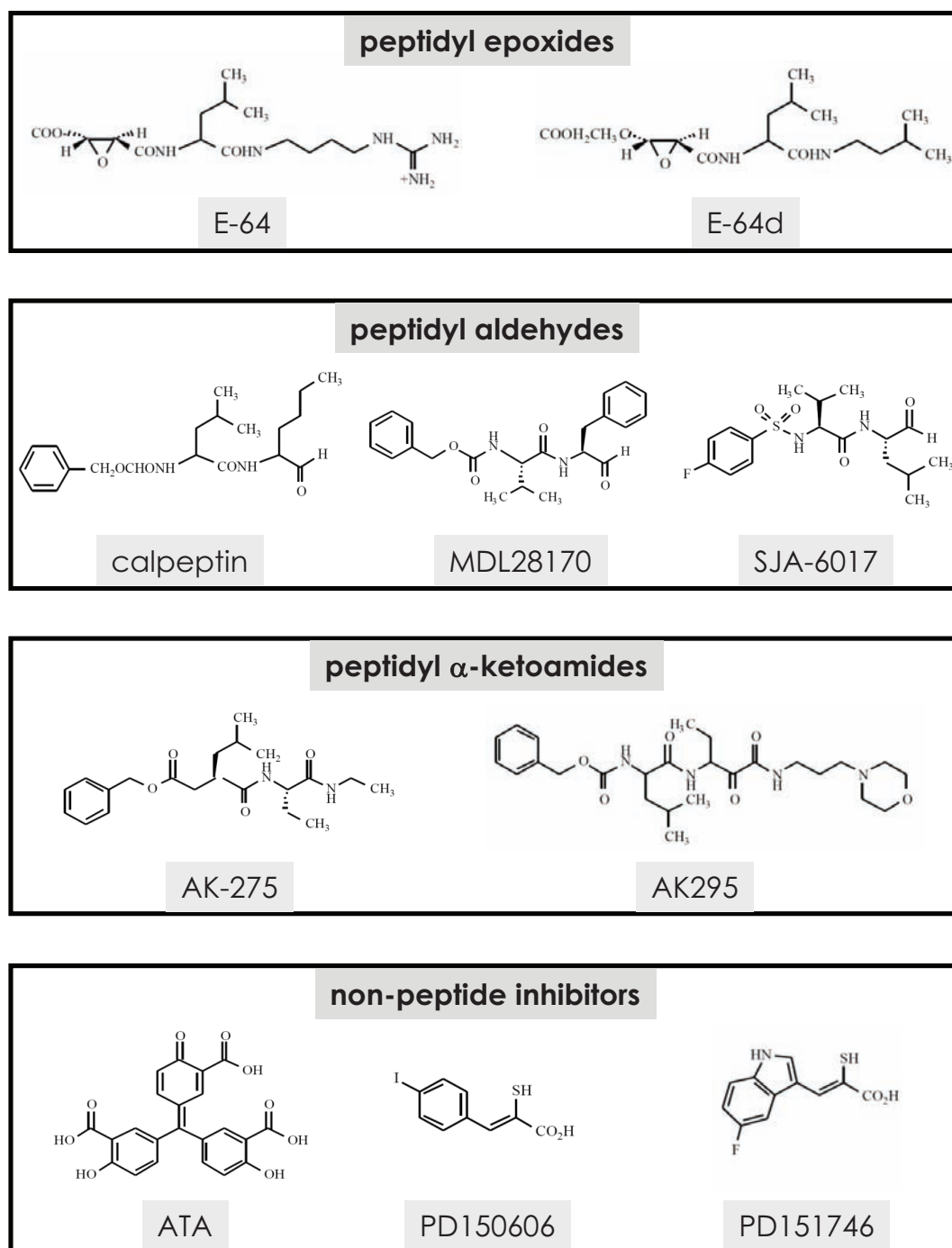
peptidyl epoxides

E-64   E-64d

peptidyl aldehydes

calpeptin   MDL28170   SJA-6017

peptidyl α-ketoamides

AK-275   AK295

non-peptide inhibitors

ATA   PD150606   PD151746

Fig. (3). Different classes of calpain inhibitors: peptidyl epoxides, peptidyl aldehydes, peptidyl α-ketoamides and non-peptide inhibitors.

In mammals, the calpain activity is controlled *in vivo* by calpastatin, a peptide that is the only specific inhibitor of the calpain family discovered to date; its specificity is determined by the simultaneous binding of three calpastatin subdomains to both subunits of heterodimeric calpains [35]. Another line of investigation have shown that nitric oxide (NO)-donors such as S-nitroso-5-dimethylaminonaphthalene-1-sulphonyl (dansyl-SNO), S-nitrosoglutathione (GSNO), (+/-)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR-3) and S-nitrosoacetylpenicillamine (SNAP) are able to block the enzyme activity of different classes of cysteine peptidases, including mammalian cal-

pains, via NO-mediated S-nitrosylation of the cysteine catalytic residue [36, 37].

4. GENES CODING FOR CALPS IN TRYPANOSO-MATIDS

In humans, a large calpain gene family is described, with at least 14 members [19]. Calpain-related gene families in non-mammalian taxa are usually small [25], but in the trypanosomatids *T. brucei, T. cruzi* and *L. major* this situation is quite distinct. In 2005, Ersfeld *et al.* [18] employed whole-genome analysis and showed the presence of a large and

diverse family of CALPs in the trypanosomatids *T. brucei, T. cruzi* and *L. major*. The authors categorized these proteins into five groups, based on their structural features. Members of groups 1 and 2 present four domains and are termed CALPs, being distinguished by their N-terminal domains: while group 1 contains the domain I^K, which is highly conserved in kinetoplastids, group 2 contains the non-conserved domain I^H. As specified earlier, sequence characterization revealed, in most CALPs, a well-conserved peptidase domain, known as domain II, although the amino acid residues critical for catalytic activity are frequently altered. The overall identity of domain II of kinetoplastids compared with conventional domain II of a catalytically active calpain is approximately 25%. In this sense, the absence of amino acid residues essential for catalytic activity and the moderate overall degree of sequence identity suggest that most CALPs do not have proteolytic activity. On the other hand, although calcium-binding motifs are absent in domain IV in both groups of CALPs, amino acid residues that are critical for binding of calcium within domain II in mammalian calpains are partially conserved in some kinetoplastid sequences. Members of group 3 are termed small kinetoplastid calpain-related proteins (SKCRPs), consisting only of the kinetoplastid-exclusive domain I^K. In common to group 1, SKRCPs may display an N-terminal dual acylation motif. Six SKRCPs were found in *T. brucei*, nine in *T. cruzi* and ten in *L. major*, with an average length of approximately 200 amino acids. This domain showed no similarities to other known proteins, so there is no indication as to its function. Groups 4 and 5 contain highly divergent CALPs not studied yet: group 4 is characterized by the presence of three repeats of domains II and III, and group 5 by N-terminal repeats of domain I and single domains II and III [18].

More recently, a molecular study aimed to better characterize the flagellar protein H49 and its repeats revealed that these proteins are members of the calpain gene family of *T. cruzi* [38]. Galetovic *et al.* [38] used the sequence of clone H49 (GenBank L09564) as a query to search for H49 genes in the *T. cruzi* genome database through the tblastn program on GeneDB. As a result, the research group found eight contigs carrying the 204-bp repeats of H49 proteins associated with the calpain-like cysteine peptidase sequences, which were referred to as H49/calpains. These proteins keep the CysPc calpain domains IIa and IIb, characteristic of calcium-dependent cytoplasmic cysteine peptidases and papain-like proteins, and among the 53 *T. cruzi* calpain-like sequences identified in the genome database performed by the authors, eight were associated with H49 sequences belonging to group IV previously described by Ersfeld *et al.* [18].

This surprising expansion of CALP genes in these parasites may be correlated to distinct situations: for instance, the need for survival or growth within several distinctive environments such as the mammalian host and the insect vector may implicate in organism-specific functions for these proteins [18, 39]. A comparative study of calpains expression in trypanosomatids with different life cycles may help to determine the general functions of these molecules in the Trypanosomatidae family, as well as its specific role for each parasite. The identification and biochemical/immunological characterization of CALPs performed in trypanosomatids are described below.

5. CALPS in *T. brucei*

Hertz-Fowler *et al.* [40] presented the first report of a member of a novel family of calpain-related genes in trypanosomatids, specifically in *T. brucei*. The cytoskeleton-associated protein is characterized by the similarity to the catalytic region of calpain-type peptidases and it is detected exclusively in procyclic form (the morphological stage detected in the invertebrate vector), being evenly distributed across the subpellicular microtubule corset. In the absence of any notable differences between the cytoskeletal organizations in the different life cycle forms of the parasite, it was suggested that this stage-regulated protein, also called CAP5.5 or TbCALP4.1, should play more complex functions, such as a role in vector-parasite mediated signal transduction, besides its structural role. This was reinforced by the detection of another CALP, TbCALP1.1, which is upregulated in the bloodstream forms of the parasite [41]. A bioinformatic analysis pointed to the presence of a family of at least nine proteins (TbCALP4.1-9) with some degree of similarity with the proteolytic domain of calpains but devoid of calcium-binding domains. Nevertheless, there is no indication whether any of *TbCALP4.2-9* genes are expressed. Although there is an overall similarity with the catalytically active domain of calpains, only one of the three amino acids constituting the active site in classical calpains (CHN) is conserved in CAP5.5 (SYN) [40].

Through RNAi experiments that selectively targeted either *CAP5.5* or its paralogue, named *CAP5.5V*, Olego-Fernandez *et al.* [39] subsequently showed that *CAP5.5* is essential for cell morphogenesis in procyclic forms, while *CAP5.5V* is expressed and essential in bloodstream forms. In addition, it was demonstrated that the paralogous genes provide analogous roles in cytoskeletal remodeling for the two life cycle stages, being necessary for the correct morphogenetic patterning during the cell division cycle and for the organization of the subpellicular microtubule corset. The authors suggested that loss of proteolytic activity may have been an important step in the functional evolution of these CALPs, mainly to act as microtubule-stabilizing proteins.

CAP5.5 has also been shown to be both myristoylated and palmitoylated, suggesting a stable interaction with the cell membrane through interactions with the subpellicular microtubule cytoskeleton [40]. Interestingly, the anti-CAP5.5 antibody labeled the whole cell body excluding the flagellum. Some of the CALPs in *T. brucei* contain these N-terminal fatty acid acylation motifs, indicating the association of these proteins with cellular membranes [40]. This N-terminal domain has been also detected in a family of small myristoylated proteins (SMPs) that are present in *Leishmania* spp., *T. brucei* and *T. cruzi*, being required for the localization of proteins in the parasite surface or in intracellular membranes [42], and it is unique to kinetoplastids [18].

Liu *et al.* [43] presented a comprehensive analysis of the expression patterns and subcellular localization of selected members of groups 1-3 [18] in *T. brucei*. When comparing the transcriptional status of distinct CALPs and SKCRPs, it was observed that two transcripts, *SKCRP5.1* and *CALP8.1/CAP5.5V*, were differentially expressed in bloodstream cells, while the transcripts *SKCRP1.6, SKCRP7.2* and *CALP4.1/CAP5.5* were differentially expressed in procyclic

forms. It is not known whether *CAP5.5* and *CAP5.5V* are functionally equivalent or sequence differences confer life cycle-specific functions. Three principle types of localization were detected: the flagellum, the cell body and the periphery of the cell body. In particular, the differential flagellar localization of some CALPs supported the view that the flagellar membrane of trypanosomes is not homogeneous but compartmentalized in order to fulfill specific functions. The precise functions of these proteins are not known, since the proteolytic activity is most likely absent, but it is hypothesized that CALPs could be involved in regulatory processes [18, 43]. Another important aspect is the detection that *SKCRP5.1* shares the *META-1* gene homologous domain with *Leishmania* spp., where META-2 protein contains the calpain domain I^K and it is up-regulated in the infective metacyclic promastigotes [44].

6. CALPS in *Leishmania* spp.

Bhattacharya *et al.* [45] showed the presence of a calcium-dependent proteolytic activity in *L. donovani* promastigote cell lysate, which was named caldonopain. The activity was more intense at pH 7.4, and 12-h incubation was required for its activation: the delayed action of the proteolytic activity in the presence of calcium was used as the basis for the identification of this particular peptidase. The enzyme was inhibited by sulfhydryl reagents and it was found to be localized in the cytosol along with a possible specific inhibitor, named caldonostatin. The same research group showed that there was a 95-kDa protein band in the cytosolic fraction that was detected in sodium dodecyl sulfate polyacrylamide gel electrophoresis containing gelatin, with its proteolytic activity enhanced in the presence of Ca^{2+} ions [46]. In addition, a similar activity was found in amastigotes [46]. However, the gene(s) or protein(s) responsible for this activity were not identified or isolated.

The investigation of changes in leishmanial gene expression between drug resistant/sensitive strains and in the up-regulation of virulence-related genes in infective forms has highlighted the presence of CALPs in the *Leishmania* genus as well as its participation in crucial pathogenic events for the parasite. When highly sensitive gene expression microarray technology was employed to identify genes that are differentially expressed in *Leishmania donovani* isolated from post kala-azar dermal leishmaniasis (PKDL) patients in comparison with those from visceral leishmaniasis, a 2-fold higher expression of five proteins in PKDL parasites was reported, including a short CALP with significant homology to a *T. brucei* CALP [47]. These results reinforced that, besides the immunological mechanisms that are able to alter the host-parasite equilibrium in favor of the latter, parasite genetic determinants also contribute to the long-term maintenance of *L. donovani* in the human host and its subsequent reactivation after clinical cure of visceral leishmaniasis, as it occurs in PKDL.

Subproteomic analysis of soluble proteins of the microsomal fraction from *L. major* promastigotes revealed the presence of 41 different proteins, including a protein designated LmjF20.2310 that presented a high level of sequence similarity to the C-terminal domain of the META-2 protein, a calpain-like paralogue that maps to *META1* gene locus,

which is a region that encodes the protein virulence factors found in vacuoles located around the flagellar pocket [44, 48]. In a similar approach, a comparative proteomics screen was performed between antimonial-resistant and -sensitive *L. donovani* strains isolated from kala-azar patients [49]. In this work, the calpain-related protein SKCRP14.1, which is down-regulated in the resistant strain, was shown to modulate the susceptibility to antimonials and miltefosine by interfering with drug-induced programmed cell death (PCD) pathways: when over-expressed, this CALP significantly increased the sensitivity of the resistant strain to antimonials, being able to promote PCD, but the opposite effect was seen in miltefosine-treated cells, in which this CALP protected against miltefosine-induced PCD. It was concluded that SKCRP14.1 is likely to be a regulator of PCD, and since an altered expression of the same protein can have such different outcomes on drug-induced PCD, there may be more than one PCD pathway in *Leishmania* spp. [49]. As a matter of fact, conflicting roles for calpain activity in contributing to the promotion and/or suppression of apoptosis have been proposed in mammals, being suggested that calpains must have a wide influence over many apoptotic processes, and their specific roles during apoptosis may differ depending on cell type and the nature of the apoptotic stimulus [19]. In trypanosomatids, it is possible that the great variety of calpains structure based on the presence of distinct domains in each group of Ersfeld's classification [18] and also highlighted by our group in this review must contribute to the variety of functions performed.

Caspase activities seem to be essential for the induction of the typical nuclear features of apoptosis, whereas they are not required, in several circumstances, for the induction and execution of PCD [50]. Evidence is now accumulating that non-caspase peptidases including cathepsins, granzymes, the proteasome complex and calpains also have roles in mediating and promoting cell death [51]. In this sense, when studying staurosporine-induced PCD in *L. major*, Arnoult *et al.* [52] found out that nuclear apoptosis-like features were prevented by cysteine peptidase inhibitors, and cell-free assays indicated that the cysteine peptidases involved in this process are possibly related to the calpain family. It was concluded that the ability of the parasite to undergo a cell death process with an apoptosis-like phenotype may play a role in favoring both parasite evasion from the host immune response, and parasite growth in the infected macrophages [52]. Corroborating this finding, calpain inhibitor I was shown to interfere with apoptotic DNA fragmentation in *L. donovani* promastigote death induced by miltefosine, but did not prevent cell shrinkage or phosphatidylserine externalization, which is suggestive that at least part of the apoptotic machinery operating in promastigotes involves proteases [53]. It is worth mentioning that calpain inhibitor I inhibits calpains, cathepsins B and L, as well as the proteasome, which implies that proteases are most likely involved in the molecular signaling leading to nuclear changes but the broad spectrum of activity of this inhibitor precludes any clear identification of its mechanism of action.

Calpain-like cysteine peptidases are described as playing a crucial role in NO-mediated cell injuries [54]. For instance, calpain activation is linked to NO-triggered excitotoxic events with the execution of caspase-independent apoptosis

in neurons [54]. Holzmuller *et al.* [55] demonstrated a rapid and extensive fragmentation of nuclear DNA in both axenic and intracellular NO-treated amastigotes of *L. amazonensis*. On the contrary, the exposure of NO-treated amastigotes with specific proteasome inhibitors, such as lactacystin or calpain inhibitor I, markedly reduced the induction of the NO-mediated PCD [55].

As the changes in the biochemistry and morphology of *Leishmania* from one life cycle stage to another are most likely the result of programmed changes in the gene expression, due to the changes in the external environment of the parasite, DNA microarray analysis was used in *L. major* in order to analyze the global changes in gene expression as procyclic promastigotes differentiate *in vitro* into metacyclic promastigotes [56]. Changes in mRNA abundance were detected for several known genes, and several hundred additional genes were identified whose expression changes during metacyclogenesis. In this sense, one CALP from *L. major* is up-regulated in the procyclic promastigote insect stage, and two distinct CALPs are up-regulated in the metacyclic insect stage. Life cycle-specific expression may also demarcate the search for specific functions of these CALPs.

Our research group started to study the presence of CALPs in *L. amazonensis* by the usage of the calpain inhibitor MDL28170, which is a potent and cell-permeable inhibitor of calpain I and II [33]. This molecule was added to replicating *L. amazonensis* promastigote forms in different concentrations, and our results showed that MDL28170 arrested the growth of *L. amazonensis* in a dose-dependent manner. The IC_{50} after 48 h was found to be 23.3 μM. The antileishmanial activity was irreversible, since protozoa pretreated for 72 h with the calpain inhibitor at 30 μM did not resume growth when sub-cultured in fresh medium. In addition, optical microscopy observations showed a massive deterioration of promastigote cells (Fig. **4**) [57].
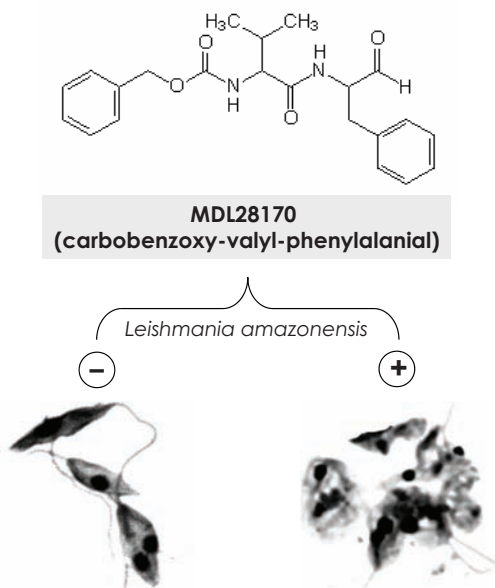

Fig. (4). Chemical structure of the calpain inhibitor MDL28170 and microscopic observations of the viability of promastigote forms of *Leishmania amazonensis* incubated in the absence (-) or in the presence of MDL28170 at 20 μM (+). Reprinted with permission [79].

When we aimed to detect calpain homologues in this protozoan by immunoblot assays using different anti-calpain antibodies, we found out that the anti-Dm-calpain antibody, raised against *D. melanogaster* calpain, strongly recognized a polypeptide band migrating at approximately 80-kDa, which suggested that this trypanosomatid possesses molecules that share antigens with invertebrate calpain-related enzymes [57]. Simultaneously, no common epitopes were found between mammalian calpains and *L. amazonensis* polypeptides [57]. Interestingly, the calpain-like molecule was detected on the cell surface of *L. amazonensis*, as demonstrated by flow cytometry and fluorescence microscopy analyses using the anti-Dm-calpain antibody (Fig. **5**) [57]. These results added new *in vitro* insights into the exploitation of calpain inhibitors in treating trypanosomatid infections and added this family of proteases to the list of potential targets for development of more potent and specific inhibitors.

7. CALPS IN *T. cruzi*

In *T. cruzi*, the detection of CALPs was initially associated to stress conditions. Giese *et al.* [58] described the identification of a *T. cruzi* (isolate Dm28c) CALP, named TcCALPx11, by microarray analysis. Its gene is a member of group 1 [18], which is the most conserved group of CALPs in these protozoa [18]. In addition, its mRNA was 2.5 times more abundant in epimastigotes (insect stage) under nutritional stress, a requirement for differentiation into metacyclic trypomastigotes (infective form), than in epimastigotes growing in complete medium. The Western blot analysis of *T. cruzi* protein extracts at various stages of differentiation, employing an antiserum against TcCALPx11, revealed a single 80-kDa protein found exclusively in epimastigotes, being suggested that the epimastigote-specific expression could implicate this CALP in the adaptation of epimastigotes to the insect vector environment [58]. Alternatively, its increased expression at the onset of metacyclogenesis is consistent with a role in the differentiation process as well as a stress-induced protein [58]. The over-production of this protein in transfected cells did not alter the morphology, the growth rates or the differentiation rates. The bioinformatics analysis gave no indication of putative acylation motifs in TcCALPx11, in contrast to the *T. brucei* CAP5.5 [40], suggesting that TcCALPx11 is not membrane-associated, although the biochemical fractionation of cells into detergent soluble and insoluble fractions showed that the protein partitioned mainly in the insoluble fraction. Finally, the absence of proteolytic activity also led to the suggestion of the role of this CALP in signal transduction.

As previously detected in *T. brucei*, CALPs were also found as microtubule-interacting proteins in *T. cruzi*. In the latter, the H49 antigen is located in the cytoskeleton of epimastigote forms, mainly in the flagellar attachment zone, and sequence analysis demonstrated that the 68-amino acid repeats are located in the central domain of CALPs belonging to group 4 [18]. Critical alterations in the catalytic motif suggest that H49 protein lack calpain proteolytic activity. The so-called H49/calpains could have a protective role, possibly ensuring that the cell body remains attached to the flagellum by connecting the subpellicular microtubule array to it [38]. Inexact H49 repeats were found in the

genomes of other trypanosomatids, including *T. brucei, L. major, L. infantum* and *L. braziliensis*, with less than 60% identity to H49 and located in CALPs, including *T. brucei* CAP5.5 [38].
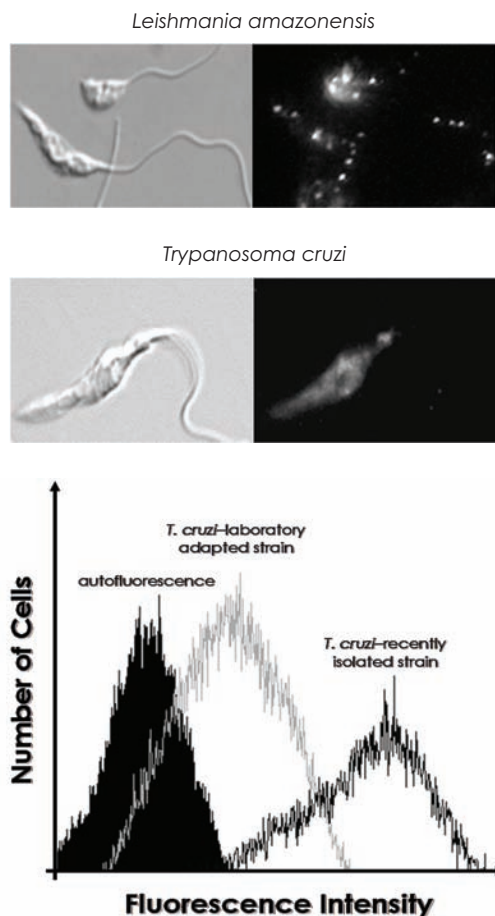

Fig. (5). Detection of binding of anti-calpain antibodies to the surface of *Leishmania amazonensis* promastigotes and *Trypanosoma cruzi* epimastigotes. Fluorescence microscopy showing the binding profile after non-permeabilized parasites incubation with anti-Dm calpain antibody followed by incubation with secondary antibody conjugated with fluorescein. Alternatively, epimastigotes regularly kept in brain heart infusion culture medium (*T. cruzi*-laboratory adapted strain) and epimastigote cells obtained from the differentiation of trypomastigotes after a blood passage in mouse (*T. cruzi*-recently isolated strain) were fixed with paraformaldehyde and incubated in the absence (autofluorescence) or in the presence of anti-Dm-calpain antibody followed by incubation with secondary antibody conjugated with fluoresceine. For simplicity, only the autofluorescence of recently isolated cells is shown, since the adapted strain presented similar values (data not shown). When treated only with the secondary-fluoresceinated antibody, both strains generated similar curves to that observed in the autofluorescence of cells (not shown). Note that laboratory-adapted strain had significant diminished expression of calpain-like molecules when compared to parasites obtained after passage in mouse. For experimental details see [57,61].

In a distinct approach, the use of proteomic analysis was employed for the identification of new therapeutic targets in *T. cruzi* [59]. The need for new options to treat Chagas' disease is determined by the limited therapeutic options, which are restricted to benznidazole and nifurtimox [10]. Taking these facts into account, the proteomic analysis of *T. cruzi* with selected *in vivo* and *in vitro* resistance to benznidazole showed that some proteins are over-expressed in resistant parasites, probably as an adaptation to the unfavorable drug stress conditions. In this sense, a CALP was found among the proteins identified in major amount in both resistant samples that were selected *in vivo*, but not *in vitro* [59]. Interestingly, no common over-expressed protein was present in the three samples that were analyzed, probably due to the wide genetic variability of the parasite, which leads to distinct susceptibilities to these compounds [60].

In a recent paper from our group, the effects of the calpain inhibitor MDL28170 were determined upon *T. cruzi* growth, and the presence of CALPs in distinct *T. cruzi* phylogenetic lineages was also determined [61]. Our results showed that the calpain inhibitor arrested the growth of *T. cruzi* epimastigote forms in a dose-dependent manner, although with higher IC_{50} values when compared to the calculated value for *L. amazonensis* [57]. MDL28170 promoted several ultrastructural alterations [62], such as disorganization of the reservosomes (which are storage organelles and the main site of protein degradation [63], intimately implicated in epimastigote into trypomastigote differentiation) [64], Golgi and plasmatic membrane [62]. Reservosomes and Golgi were severely affected, showing a washed-out appearance with loss of organelles' electrondensity and complete disruption of their membranes [62].

As it occurs with *L. amazonensis* [57], *T. cruzi* possesses molecules that share antigens with invertebrate calpain-related enzymes, such as *D. melanogaster* CALP (Fig. **5**). Simultaneously, the absence of cross-reactivity between calpain-like molecules in *T. cruzi* and anti-human m-calpain [61] is suggestive of substantial structural differences among these molecules, as previously detected by Ersfeld *et al.* [18]. We have also presented evidence that the maintenance of *T. cruzi* in axenic culture for a long time led to a decrease in the expression of CALPs (Fig. **5**), which may suggest that there is a direct relationship between the expression of calpain-like molecules and the parasite virulence [61], as it has been previously reported that the loss of virulence is associated to modifications of biological properties of this parasite that might lead to changes in the expression of some proteins [65]. Additionally, our results also demonstrated a direct correlation between the relative levels of CALPs detection and *T. cruzi* strains classification in the three major phylogenetic groups (TCI, TCII and Z3), so it is possible that modulation in the expression of these proteins does take place. In our work, *T. cruzi* CALPs that cross-react with anti-CAP5.5 are located mainly in the intracellular milieu, which may indicate a possible correlation regarding the location of a subset of CALPs in both trypanosomes [61].

Fluctuations of the expression levels of calpain-related molecules were also observed when Dm28c cells were treated with MDL28170 at IC_{50}: a reduced labeling was found in treated cells, and a simultaneous increase in cruzipain expression was detected [61]. Cruzipain is the major cysteine peptidase in *T. cruzi*, being expressed at different levels by distinct parasite stages, and many evidences point to its participation in penetration into the host cell, escape

from the immune system and in the differentiation processes [66]. The possible correlation of the expression levels of cysteine peptidases in *T. cruzi* may suggest that the over-expression of a certain cysteine peptidase may be necessary in order to titrate out the toxic levels of the inhibitor compound that is active against a different peptidase from the same class [67]. In this sense, when the effects of pepstatin A, a powerful aspartic peptidase inhibitor, on *T. cruzi* were explored, the proliferation of epimastigote forms was arrested and significant morphological alterations were found. In addition, pepstatin A induced an increase of 54% and 98%, respectively, in the surface expression of gp63- and calpain-related molecules in epimastigotes, but not in the cruzipain level [68].

The blockage of calpain molecules by the anti-calpain antibody led to a significant reduction in the capacity of *T. cruzi* epimastigote adhesion to the insect guts of the insect vector *Rhodnius prolixus* in a dose-dependent manner, the inhibition ranged from 30% to 60% as antibody concentration rose from 1:250 to 1:50 [62]. On the other hand, parasites treated with the pre-immune serum at the highest concentration adhered to the guts at a rate similar to that of the control [62]. The calpain inhibitor MDL28170 also reduced significantly the number of parasites adhered to the insect luminal midgut surface [62]. Collectively, these results pointed out to the participation of CALPs on the epimastigote-insect vector interaction process. Nevertheless, it could be assumed that calpains could participate in the aforementioned events through a non-catalytic mechanism, as demonstrated in other cellular systems [69]. Moreover, a first pre-requisite for CALPs to act on parasite binding to the insect midgut would be its surface location. Immunocytochemistry images showed scarce fields of membrane labeling with anti-calpain antibodies. Bioinformatics analysis gives indication of putative acylation, myristoylation and palmitoylation motifs in *T. cruzi* calpains, suggesting that they may be membrane-associated [58]. Accordingly, TcCALPx11 (XP_816697.1) partitioned in the insoluble fraction after detergent extraction, suggesting an association with membranes [58]. Additionally, calpains were identified in a proteomic analysis of detergent-solubilized membrane proteins from *T. cruzi* [70].

MDL28170 was also capable of significantly reducing the viability of bloodstream trypomastigotes, presenting an IC_{50}/24 h value of 20.4 μM [62, 71]. Also, parasites pre-treated with the inhibitor, at sub-inhibitory drug concentrations, prior to macrophage infection presented a clear dose-dependent inhibition profile of this cellular interaction. In addition, macrophages experimentally infected with *T. cruzi* trypomastigote forms that were post-treated with the calpain inhibitor presented a significant reduction in the percentage of intracellular amastigotes, resulting in a diminished infection [71]. Taken together, these findings robustly confirmed that the calpain inhibitor MDL28170 acted against *T. cruzi* clinically relevant forms, trypomastigotes and amastigotes, without displaying any relevant cytotoxic effect on mammalian host cells [71]. Interestingly, MDL28170 arrested the *in vitro* epimastigote into trypomastigote differentiation, presenting a time and dose-dependent inhibition profile in the *T. cruzi* metacyclogenesis [62].

8. CALPS IN MONOXENIC TRYPANOSOMATIDS

Monoxenic trypanosomatids, a group of protozoa that by definition infects invertebrates only, have been used routinely as laboratory models for biochemical and molecular studies because they are easily cultured under axenic conditions [1], and they contain homologues of virulence factors from the classic human trypanosomatid pathogens [72, 73]. Our research group became involved in the study of CALPs though the purification of a proteolytically active cysteine peptidase in the culture supernatant of the insect trypanosomatid *Crithidia deanei* that shares some features with calpains [74]. The enzyme was purified as a homotrimer of an 80-kDa protein, and it exhibited maximal activity at pH 7.0. Its proteolytic activity was completely blocked by the cysteine peptidase inhibitor E-64 and greatly stimulated in the presence of dithiothreitol. In addition, the complete abolition of proteolytic activity in the presence of EGTA indicated the absolute requirement of Ca^{2+} for the enzymatic activity. The apoenzyme had its activity restored in the presence of milli-molar levels of Ca^{2+}, which confirmed our results. In order to test whether anti-calpain antibodies cross-react with the Ca^{2+}-dependent cysteine peptidase from *C. deanei*, the purified enzyme was blotted and probed with the following polyclonal antibodies named C21, C22 and C23, raised against the whole molecule, the cysteine active site and the histidine active site, respectively, of human brain m-calpain [75], as well as with anti-Dm-calpain antibody raised against *Drosophila melanogaster* calpain [76]. No common epitopes were found between mammalian calpains and *C. deanei* purified calpain-like enzyme, but the anti-Dm calpain antibody cross-reacted with it. Although some degree of similarity was displayed to invertebrate calpain-related enzymes, we cannot state that this enzyme is a true calpain family member, since its microsequencing is not available. In addition, its physiological function was not addressed yet.

Recently, our group has demonstrated that an 80-kDa CALP that cross-reacted with the same anti-Dm calpain antibody was detected in both the cellular body and flagellum of *Herpetomonas samuelpessoai* promastigote cells, and its presence is enhanced in dimethylsulfoxide (DMSO)-induced paramastigote cells [77]. Interestingly, an additional 30-kDa calpain-related polypeptide was exclusively observed in the latter cells. The *Herpetomonas* genus represents an interesting model to study cellular differentiation, since it displays three developmental stages during its life cycle - promastigote, paramastigote and opisthomastigote [1]. In *H. samuelpessoai*, the cellular differentiation from promastigotes into paramastigotes/opisthomastigotes occurs in the stationary phase, and also by high incubation temperature (37°C) or after exposition to metabolic inhibitors, including DMSO [78]. We may conclude that there may be similarities in the epitopes defined by the polyclonal anti-Dm calpain and trypanosomatid calpain-related proteins, and that differentiation of these microorganisms is correlated with the altered expression of distinct CALPs.

CONCLUSIONS

This review provided a framework indicating that the study of calpains and/or CALPs inhibition may be an attractive anti-trypanosomatid approach irrespective of whether

these proteins are proteolytically active or not. As the up-regulation of several members of the calpain family is involved in a diverse range of biological processes and human diseases, this family of peptidases has an important therapeutic potential, and a huge effort has been made in the field of research to develop a means of identifying selective calpain inhibitors [31]. In the immediate term, further studies in trypanosomatid calpains may employ existing drugs developed for human calpains inhibition, since extreme biochemical selectivity may not be necessary for anti-protozoan drugs because of the inherent biologic selectivity in the function and location of protozoan peptidases [79]. As well, the inhibitor concentration necessary to chemically knockout a parasitic enzyme is likely much lower than that predicted for homologous host enzymes [80]. In the long term, knowledge of structural and functional relationships and substrate specificity of these proteins in trypanosomatids should make them candidates for computational-assisted drug design.

CONFLICT OF INTEREST

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

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ABBREVIATIONS

AK-275	=	Z-Leu-aminobutyric acid-CONH-CH$_2$CH$_3$
AK-295	=	Z-Leu-aminobutyric acid-CONH(CH$_2$)$_3$-morpholine
ATA	=	Aurintricarboxylic acid
CALPs	=	Calpain-like proteins
Calpeptin	=	N-Benzyloxycarbonyl-L-leucylnorieucinal, Z-Leu-Nle-CHO
dansyl-SNO	=	S-nitroso-5-dimethylaminonaphthalene-1-sulphonyl
DMSO	=	Dimethylsulfoxide
E-64	=	*Trans*-epoxysuccinyl-L-leucylamido-4-guanidino-butane
E-64d	=	(2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester
GSNO	=	S-nitrosoglutathione
kDNA	=	Kinetoplast DNA
MDL28170	=	Carbobenzoxy-valinyl-phenylalaninal, Z-Val-Phe-CHO
NO	=	Nitric oxide
NOR-3	=	(+/-)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide
PCD	=	Programmed cell death
PD150606	=	3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid
PD151746	=	3-(5-Fluoro-1H-indol-3-yl)-2-mercapto-2-propenoic acid
PKDL	=	Post kala-azar dermal leishmaniasis
SJA-6017	=	4-Fluoro-benzenesulfonyl-valinyl-L-leucinal
SKCRPs	=	Small kinetoplastid calpain-related proteins
SMPs	=	Small myristoylated proteins
SNAP	=	S-nitrosoacetylpenicillamine

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