

# Aspartic Peptidases of Human Pathogenic Trypanosomatids: Perspectives and Trends for Chemotherapy

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**Abstract:** Aspartic peptidases are proteolytic enzymes present in many organisms like vertebrates, plants, fungi, protozoa and in some retroviruses such as human immunodeficiency virus (HIV). These enzymes are involved in important metabolic processes in microorganisms/virus and play major roles in infectious diseases. Although few studies have been performed in order to identify and characterize aspartic peptidase in trypanosomatids, which include the etiologic agents of leishmaniasis, Chagas' disease and sleeping sickness, some beneficial properties of aspartic peptidase inhibitors have been described on fundamental biological events of these pathogenic agents. In this context, aspartic peptidase inhibitors (PIs) used in the current chemotherapy against HIV (e.g., amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir) were able to inhibit the aspartic peptidase activity produced by different species of *Leishmania*. Moreover, the treatment of *Leishmania* promastigotes with HIV PIs induced several perturbations on the parasite homeostasis, including loss of the motility and arrest of proliferation/growth. The HIV PIs also induced an increase in the level of reactive oxygen species and the appearance of irreversible morphological alterations, triggering parasite death pathways such as programmed cell death (apoptosis) and uncontrolled autophagy. The blockage of physiological parasite events as well as the induction of death pathways culminated in its incapacity to adhere, survive and escape of phagocytic cells. Collectively, these results support the data showing that parasites treated with HIV PIs have a significant reduction in the ability to cause *in vivo* infection. Similarly, the treatment of *Trypanosoma cruzi* cells with pepstatin A showed a significant inhibition on both aspartic peptidase activity and growth as well as promoted several and irreversible morphological changes. These studies indicate that aspartic peptidases can be promising targets in trypanosomatid cells and aspartic proteolytic inhibitors can be benefic chemotherapeutic agents against these human pathogenic microorganisms.

**Keywords:** Alternative chemotherapy, aspartic peptidases, Chagas' disease, HAART, HIV, HIV peptidase inhibitors, *Leishmania*, leishmaniasis, pathogenesis, peptidases, proteolytic inhibitors, *Trypanosoma*, virulence.

## 1. THE TRYPANOSOMATIDAE FAMILY

The Trypanosomatidae family (order Kinetoplastida) comprises a large group of flagellate parasitic protozoa that causes infections in humans, animals and plants [1–3]. Individuals of this family have a ubiquitous distribution in nature and are easily distinguished from other protozoa by their unique ultrastructure, which exhibits a net of subpellicular microtubules [4], located below the cytoplasmic membrane, firmly attached to the inner surface of the membrane, accounting for the typical morphology, as well as a unique mitochondrion, branched throughout parasite cytoplasm,

presenting a typical region of DNA condensation, known as kinetoplast, a giant network of thousands of concatenated circular mitochondrial DNAs (kDNA) [4].

A huge variety of lifestyles and adaptations to parasitism are observed in representatives of this family, as well as in the entire Order, which constitutes a very ancient group of organisms in the phylogenetic tree of eukaryotes. Individuals of this family are divided into two major groups according to their ability to infect vertebrate and/or invertebrate hosts. The heteroxenic group comprises parasites that, during their life cycle, interact with both invertebrate and vertebrate hosts, while parasites of the monoxenic group typically interact solely with invertebrate hosts. Classically, the taxonomy of trypanosomatids is defined by lifestyle, host, clinical manifestation (if applicable), and typical morphotypes. The morphotypes are defined by the position of the kinetoplast

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relative to the nucleus and the point at which the flagellum emerges from the parasite cell (Fig. 1). The distinct morphological stages are closely correlated to the genera and host-specific stages of trypanosomatids (Fig. 1) [5, 6].

Few members of this family are responsible for major important human diseases, which are collectively referred as the most neglected human diseases by the World Health Organization (WHO) (Table 1). These diseases are Chagas' disease and sleeping sickness, caused respectively by *Trypanosoma cruzi*, in South and Central America, and *Trypanosoma brucei*, in Africa; as well as leishmaniasis, caused by many species from the *Leishmania* genus, present in tropical and subtropical regions of the world (Fig. 2) [3, 7–12]. Infections in humans occur primarily through blood-sucking insects, such as triatomines, in the case of *T. cruzi*, tsetse flies for *T. brucei* and different phlebotomine sand flies species for the *Leishmania* genus [7–12]. The spread of these diseases all over the world, to many developed, non-endemic countries, is related to the globalization process and the movement of unknowingly infected people (Fig. 2).

According to WHO, in 2010 an estimated 10 million people were infected by *T. cruzi* and roughly 100 million were at risk of the disease worldwide, mostly but not restricted to Latin America. It was estimated that more than 10,000 individuals died of Chagas' disease in 2008. For leishmaniasis, in 2010, 350 million people were considered at risk of contracting the disease, and about 2 million cases occur annually, of which 0.5 million correspond to visceral leishmaniasis (Table 1 and Fig. 2) [13].

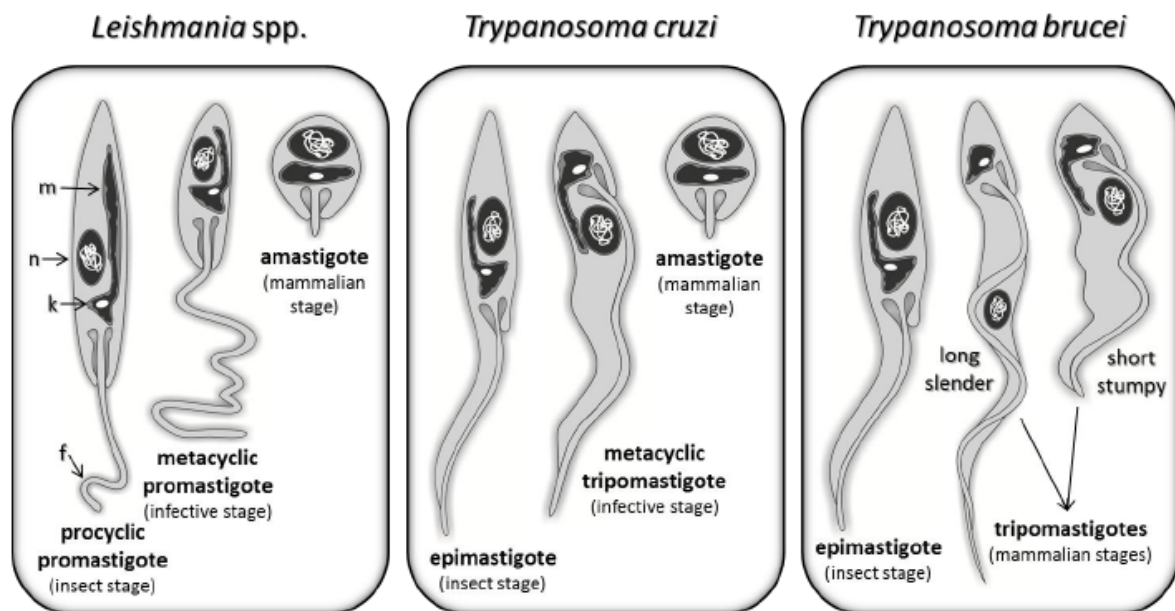
Although affecting many people around the world, the major diseases caused by parasites from the Trypanosomati-

dae family have no efficient treatment or vaccination. The available drugs (Table 1) are expensive, toxic and many parasites have already developed resistance to the chemotherapy, resulting in an urgent need to identify new targets for therapeutic alternatives [7, 8, 11, 14–16]. In this sense, this review will describe the current knowledge on trypanosomatids' aspartic peptidases and their inhibitors, since there is substantial data indicating that they can be a promising target for chemotherapy.

## 2. PEPTIDASES

Peptidases, proteinases or proteases are enzymes that catalyze the hydrolysis of peptide bonds or, in other words, proteins able to hydrolyze other proteins or peptides. These enzymes were initially classified into exopeptidases or endopeptidases according to the reaction catalyzed. Exopeptidases are capable of hydrolyzing peptide bonds at the ends of a polypeptide chain, releasing single amino acid, dipeptide or tripeptide residues, while endopeptidases preferentially act on peptide bonds in the inner regions of a polypeptide [17, 18].

The availability of structural and mechanistic information on these enzymes led to improvements on the classification schemes. According to the nature of the catalytic site, peptidases can be classified as aspartic, cysteine, metallo, serine, threonine, glutamic and asparagine type [17–19]. The intensive research on peptidases generates a wide amount of information, requiring a system of classification for the comprehensive study of this diversity. Recently, a new method of classification was introduced and can be easily accessed in the MEROPS database server [19]. In this system, peptidases



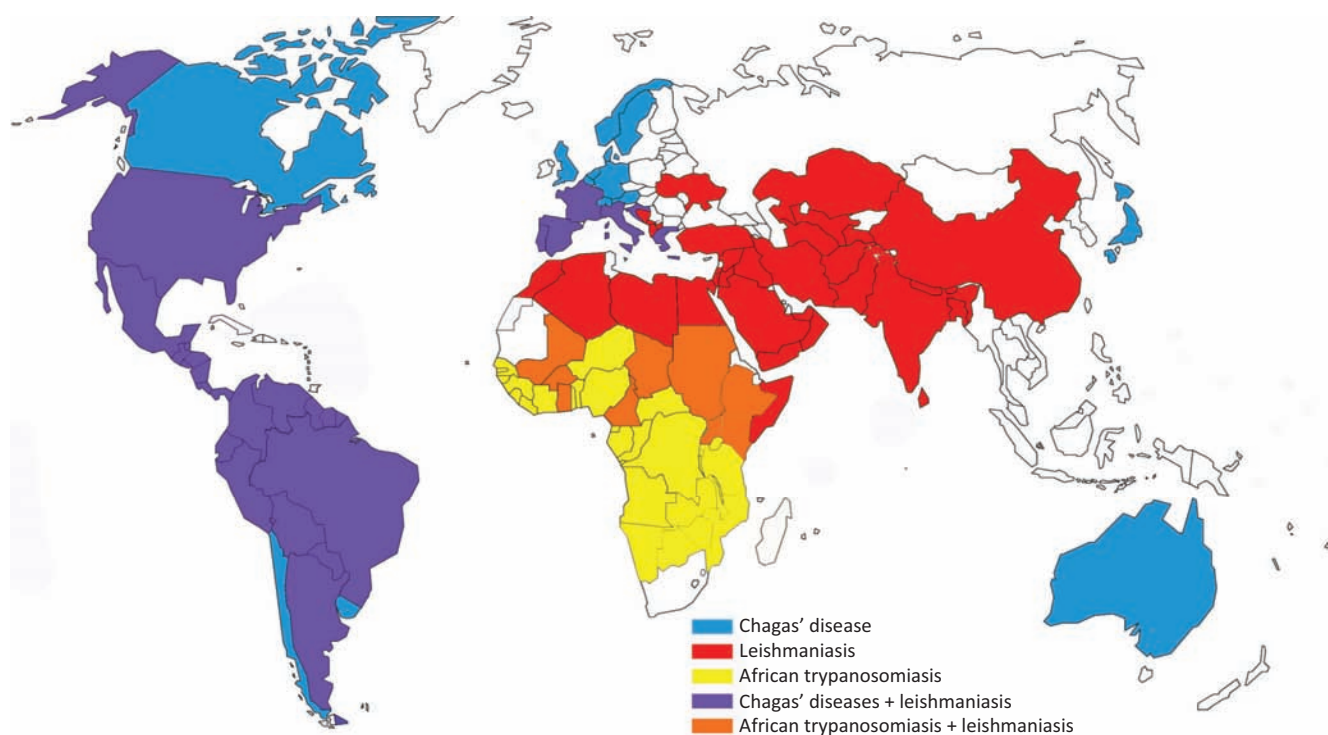
**Fig. (1).** Trypanosomatid morphotypes from *Leishmania* spp, *Trypanosoma cruzi* and *Trypanosoma brucei*. Trypanosomatid forms are defined by cell shape, point of flagellum (f) emergence and position of the kinetoplast (k) in relation to the nucleus (n). For epimastigote, promastigote and amastigote forms, the kinetoplast is located in an anterior position relative to the nucleus, while for trypomastigote form the kinetoplast is located in a posterior position. Epimastigote and trypomastigote forms present an undulating membrane connecting to their flagella, once the flagellum is attached to the cell membrane, while other forms have a free flagellum, with the exception of amastigote forms, which presents a very small flagellum. In all morphotypes, only one large mitochondria (m) is observed, which is ramified through the cell body, being the region where the kinetoplast is located.

**Table 1. Diseases Caused by Trypanosomatids of Human Medical Importance**

Trypanosomatid	Disease	Vector	People at risk (millions)	Prevalence (millions)	Available chemotherapy
<i>Leishmania</i> spp.	cutaneous, mucocutaneous or visceral leishmaniasis	phlebotomine sand fly	350	12	amphotericin B, miltefosine, paromomycin, sodium stibogluconate, meglumine antimoniate
<i>Trypanosoma cruzi</i>	Chagas' disease	triatomine	100	10	nifurtimox, benznidazole
<i>Trypanosoma brucei</i>	human sleeping sickness	tsetse fly	70	0,03*	pentamidine, suramine, melarsoprol, eflornithine, nifurtimox

\* Although 30,000 cases are reported annually, WHO estimates that about 300,000 infected individuals remain ignored in the field, due to difficulties in diagnosis and remoteness of affected areas.

### Global distribution of human infections caused by trypanosomatids



**Fig. (2).** Geographic distribution of cases reported for African trypanosomiasis, Chagas' disease and leishmaniasis around the world. Data collected from the WHO web site (<http://www.who.int/en>).

of the different classes can be further grouped into families on the basis of statistically significant similarities in amino acid sequence. For nomenclature, each family is identified by a letter that represents the catalytic domain, where A is used for aspartic type, C for cysteine type, M for metallo type, S for serine type, T for threonine type, G for glutamic type, N for asparagine type and U for unknown type; followed by a characteristic number. Families that are thought to be homologous, and had arisen from a single evolutionary origin, are grouped together in a clan. It represents one or more families that show evidence on evolutionary relationship by similar tertiary structures, order of catalytic site residues in the polypeptide chain and their common sequence

motifs around the catalytic site. For clan representation, two letters are used, being the first related to the family [19–22].

Peptidases, among many other molecules, have been evaluated with respect to its potential as new therapeutic targets. Central roles in physiological processes are carried out by peptidases, which can be found in all domains of life: Eukarya, Bacteria and Archaea, as well as in virus [23]. The importance of peptidases in biological systems is easily recognized, since all proteins of a cell need to be proteolytically processed and/or degraded at some point of cell development. The regulation of protein localization, mobility and activity, as well as modulation of protein-protein interactions, contribution for cellular information processing, gen-

eration, transduction and amplification of molecular signals are all coordinated by peptidase activity [23].

In infectious organisms, peptidases play crucial roles as virulence factors, besides its involvement in basic cellular functions. For instance, peptidases are necessary for colonization, invasion, dissemination and evasion from the host immune system [23, 24]. There are a number of excellent reviews on the functions and exploitation of trypanosomatids' peptidases as chemotherapeutic targets. Cysteine and metallo-type peptidases are the most abundant and well-studied peptidases in trypanosomatids, followed by serine peptidases. In (Fig. 3), we can see the distribution of the peptidase classes in *L. braziliensis*, *T. cruzi* and *T. brucei*.

### 3. ASPARTIC PEPTIDASES

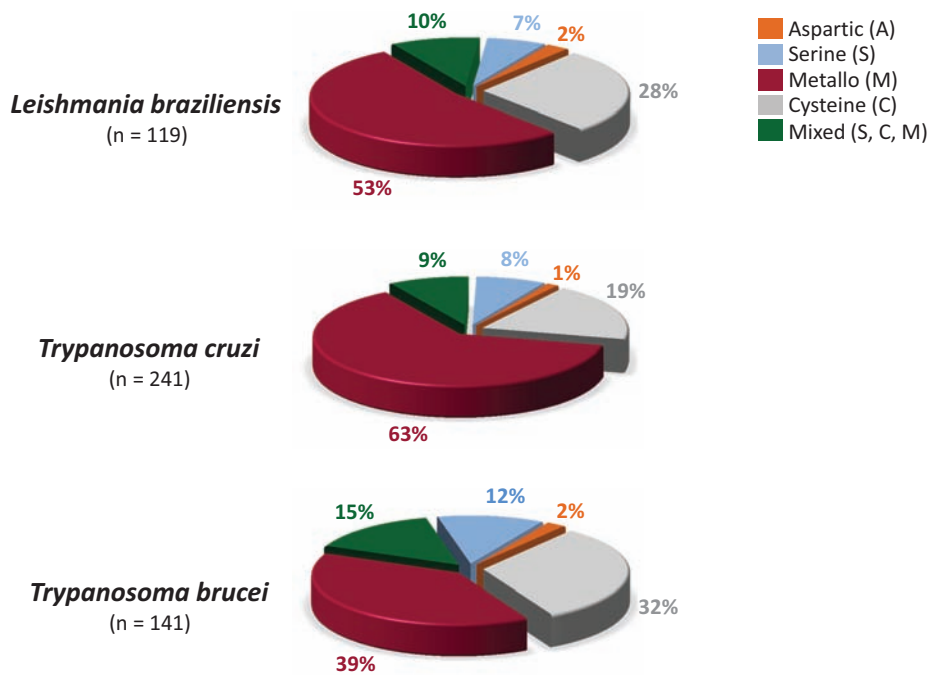
The aspartic peptidases are endopeptidases (Fig. 4) present in a wide range of organisms: vertebrates, plants, fungi, protozoa, prokaryotes and retroviruses [25-27]. The aspartic peptidases have attracted intense attention in the scientific community because of their potential for application in the food industry and as a therapeutic target for important human diseases [24, 28]. These include pepsin in peptic ulcer disease, renin in hypertension, plasmepsins in malaria, cathepsin D in metastasis of different types of cancer cells, human immunodeficiency virus (HIV) peptidase in acquired immune deficiency syndrome (AIDS), and  $\beta$ -site amyloid precursor protein cleaving enzyme (BACE) in Alzheimer's disease [24, 29-34].

Most of the aspartic peptidases are characterized by two aspartic acid carboxyl groups as key catalytic groups at their active site (Fig. 5) [35-37], three-dimensional structure similarity, low optimal pH value for best hydrolytic activity, and

a scission preference between large and hydrophobic amino acids [38-40]. Although most aspartic peptidases fit in these characteristics, considerable differences exist in terms of catalytic properties, cellular localization, biological functions and inhibition by the microbial peptide pepstatin A, which is a prototype inhibitor of aspartic peptidases (Fig. 6) [41].

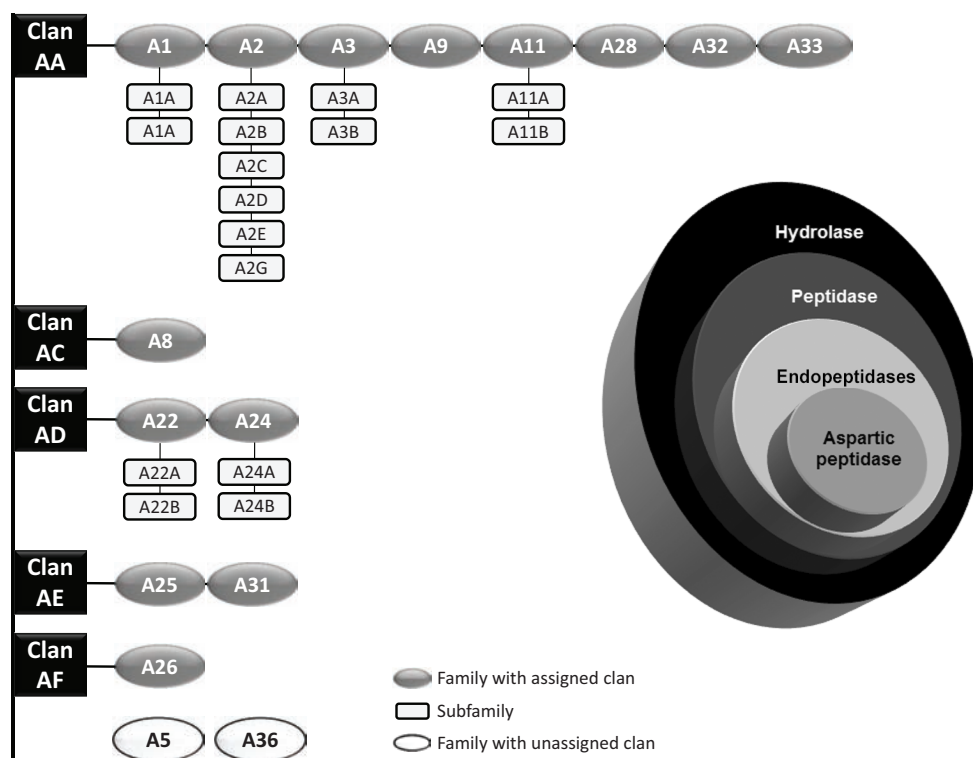
Overall, the aspartic peptidases are synthesized as inactive precursors, which are converted to the active form of the enzyme by acid-triggered, autocatalytic proteolysis and removal of lengths of polypeptides chains that are N-terminal extensions [41, 42]. Most eukaryotic aspartic peptidases are monomeric and consist of a single polypeptide chain that forms two similar domains with the active site cleft located between them; each domain provides an aspartic acid carboxyl group as key catalytic group to form the active site. In contrast, retroviral aspartic peptidases are dimeric, consisting of two identical subunits, each roughly equivalent to one domain of a eukaryotic aspartic peptidase [25-27, 29, 38, 43-45].

In the active enzyme, the two aspartic acid residues are geometrically closer and one aspartate is ionized, whereas the second one is unionized at the optimal pH [46-47]. The most widely accepted mechanism of action of the aspartic peptidases is an acid-base catalysis, which may be called a "push-pull" mechanism involving two active aspartic acid residues in the active site and a water molecule that resides between them. These two aspartic acid residues act as a proton donor and acceptor, to catalyze the hydrolysis of peptide bonds in substrates. The water molecule is partly activated by an aspartate and makes a nucleophilic attack at a specific carbonyl carbon in the substrate. The carbonyl oxygen, in turn, captures a proton from another aspartic acid in the active site, resulting in a noncovalent neutral tetrahedral

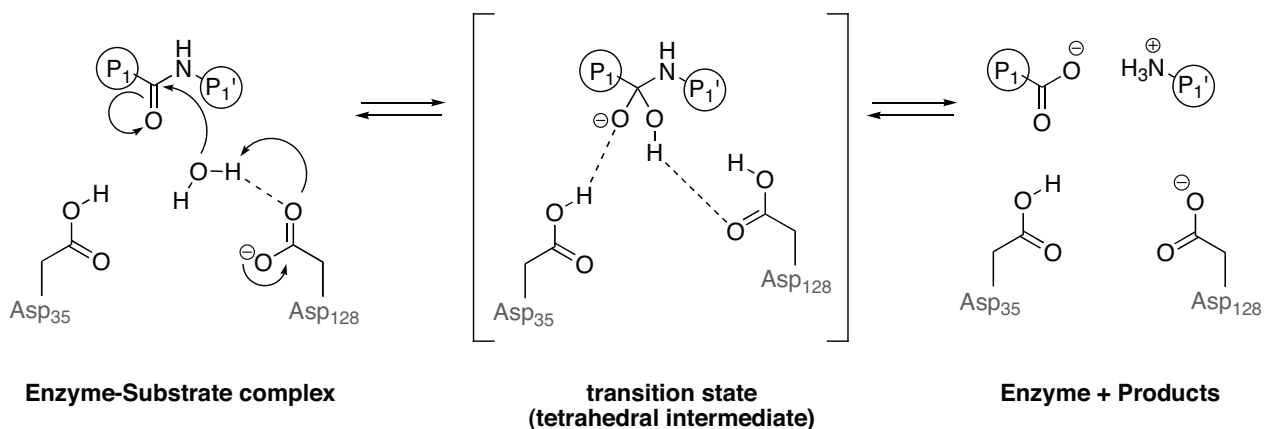


**Fig. (3).** Distribution of the different peptidase catalytic types in three representative species belonging to the Trypanosomatidae family: *Leishmania braziliensis*, *Trypanosoma cruzi* and *Trypanosoma brucei*. The data was extracted from MEROPS - The Peptidase Database [19], release 9.6; URL: <http://merops.sanger.ac.uk/>





**Fig. (4).** Classification of aspartic peptidases according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. Aspartic peptidases (EC 3.4.23.X) are ordered in subgroup 4 (peptidases) of group 3 (hydrolases) (figure on the right side). Overview of the aspartic peptidase clans, families and subfamilies according to the MEROPS Database [19]. The clan (black boxes) contains enzymes that have arisen from a single evolutionary origin of peptidases and represents one or more families (dark grey circles) that show evidence of their evolutionary relationship. The white circles represent two families with unassigned clans. In addition, some families are divided into subfamilies (light grey boxes) since there is evidence of a very ancient divergence within the family.

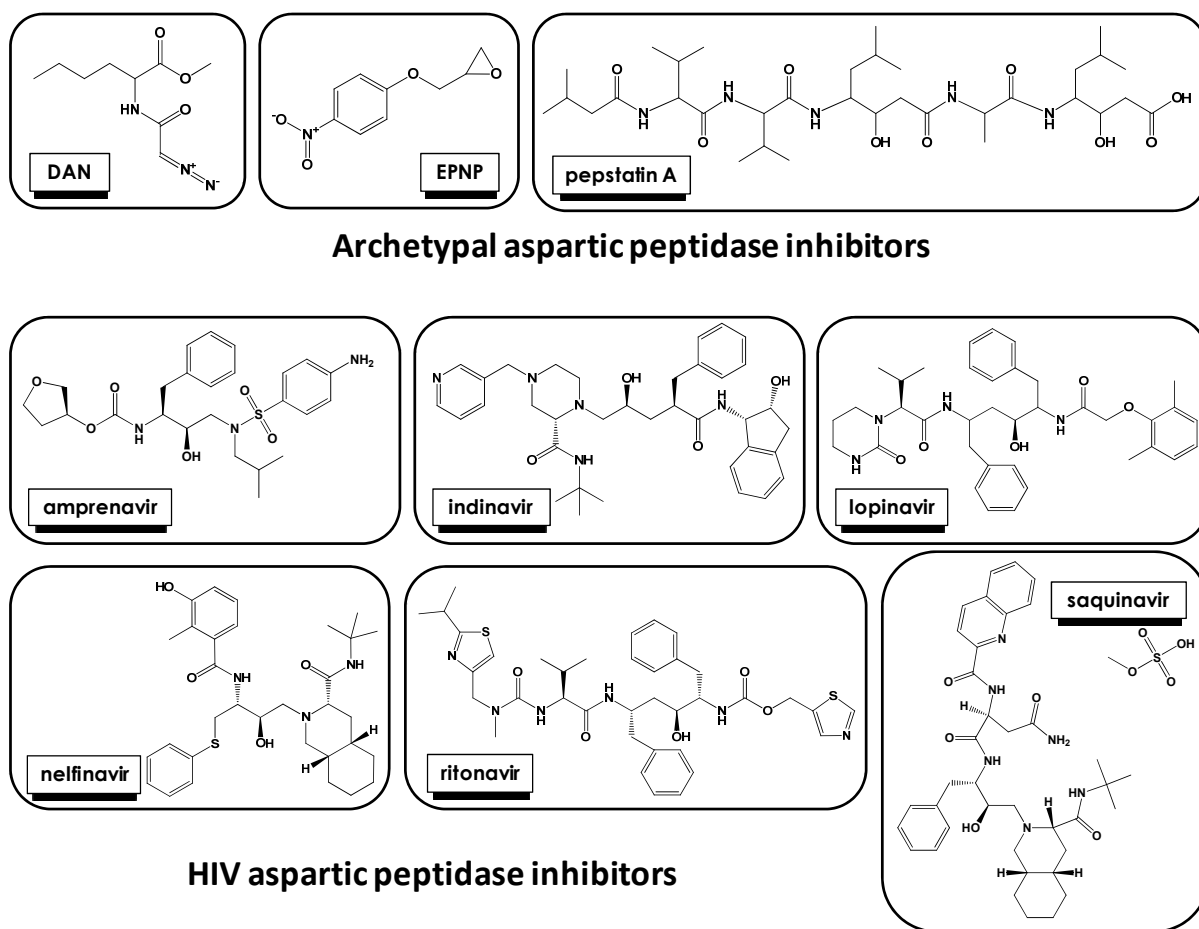


**Fig. (5).** Catalytic mechanism of aspartic peptidase proposed by Nguyen and colleagues 2008 [52]. The water molecule is partly activated by an aspartate and makes a nucleophilic attack at a specific carbonyl carbon in the substrate. The carbonyl oxygen captures a proton from another aspartic acid in the active site, resulting in a tetrahedral intermediate (transition state). Restabilizing from the transition state, the amino moiety from the substrate becomes a better leaving group, and the substrate is cleaved. Dashed lines indicate hydrogen bonds.

intermediate. This intermediate is the crucial transition state. Restabilizing from the transition state, the amino moiety from the substrate becomes a better leaving group, and the substrate is cleaved (Fig. 5) [47-52].

The aspartic peptidases are hierarchically classified into five distinct clans (AA, AC, AD, AE and AF), according to the MEROPS database. There are sixteen different peptidase

families, of which two have not been assigned to any of the existing clans, and sixteen subfamilies belonging to clans AA (twelve subfamilies) and AD (four subfamilies) [17-22, 53]. An organogram of families and clans of aspartic peptidases, focus of this review article, can be seen in (Fig. 4). In addition, the chemical structure of potent aspartic peptidase inhibitors can be seen in (Fig. 6).



**Fig. (6).** Aspartic peptidase inhibitors effective against human pathogenic trypanosomatids.

The aspartic peptidases found in the Trypanosomatidae family belong to clans AA (family A28) and AD (families A22A and A22B) (Table 2, Fig. 4). Clan AA contains the classical aspartic peptidases and clan AD comprises aspartic peptidases that hydrolyze peptide bonds within biological membranes. Clan AA is further divided into eight families, including A1 family, whose members are all-beta proteins consisting of two similar beta barrel domains, which both contribute to the formation of the active site [54], and the A2 family that is composed of proteins containing a single beta barrel domain, so dimerization must occur to form an active peptidase [55]. Family A1 contains pepsin-like enzymes such as pepsin, gastricin, rennin, cathepsin D and E, plasmepepsins (PMs), and histo-aspartic peptidase (HAP). The family A2, also termed the retropepsin family, includes HIV retropepsin [19, 21, 22, 53, 54]. In clan AD, all members have transmembrane domains that are presumed to be helical, so the protein fold must be different from the all-beta folds found in members of clan AA. Unlike members of clan AC, which also contains membrane-bound proteins, the active site is on the cytoplasmic side of the cell membrane [56]. Presenilin (A22 family), representative of clan AD, forms the catalytic core of the gamma-secretase complex required for intramembrane proteolysis of type I transmembrane proteins such as the amyloid precursor protein [28, 57]. An analysis of the occurrence of aspartic peptidase families in distinct taxonomic groups reveals interesting information, for instance, family A24 (clan AD) are found

almost exclusively in Archaea and Bacteria. Family A22 (clan AD), which is found in trypanosomatids, is still undetected in Bacteria, while A28 family (clan AA) is restricted to Eukarya (Table 3).

Although some aspartic peptidases have been identified in members of the Trypanosomatidae family, based on different properties, including molecular-level criteria, such as the reaction catalyzed, the chemical mechanism of catalysis, and the homology relationships revealed by sequence and structure similarity analyses, very little is known about the evolutionary history of aspartic peptidases in this group of parasites. However, the phylogenetic relationship of aspartic peptidase members of the A28 and A22 families recognized by the MEROPS database shows that each family (or sub-family) displays distinct evolutionary histories among distantly related eukaryotic lineages (as one may expect). In addition, trypanosomatid enzymes are consistently separated in two different groups, which indicate that the most recent common ancestor of aspartic peptidases of the A28 and A22 families in *Trypanosoma* and *Leishmania* genera are not the same (Fig. 7).

Up to now, the products of the aspartic peptidase genes in trypanosomatids were poorly or indirectly characterized, either by demonstration of degradation of aspartic peptidase substrates in crude extracts followed by inhibition by selective aspartic peptidase inhibitors (Fig. 6), or through the demonstration of the effect of these inhibitors on parasite

**Table 2.** List of the Aspartic Peptidases from Three Representatives of the Trypanosomatidae Family, Showing their Respective ID, Family and Clan

Trypanosomatid	Aspartic peptidase classification		
	Clan	Family*	MEROPS ID
<i>Leishmania braziliensis</i>	AA	A28	MER242455
	AD	A22(A)	MER184693
	AD	A22(B)	MER124926
<i>Trypanosoma cruzi</i>	AA	A28	MER242463
	AD	A22(A)	MER049314
	AD	A22(B)	MER054238
<i>Trypanosoma brucei</i>	AA	A28	MER242469
	AD	A22(A)	MER049280
	AD	A22(B)	MER048243

\* Family prototypes: A28 - DNA-damage inducible protein 1 (ddi-1) (*Saccharomyces cerevisiae*); A22 - presenilin 1 (*Homo sapiens*). Source: MEROPS - The Peptidase Database (Rawlings *et al.*, 2012), release 9.6; URL: <http://merops.sanger.ac.uk/>

**Table 3.** Distribution of Aspartic Peptidase Families from clans AA and AD Among Distinct Taxonomic Groups

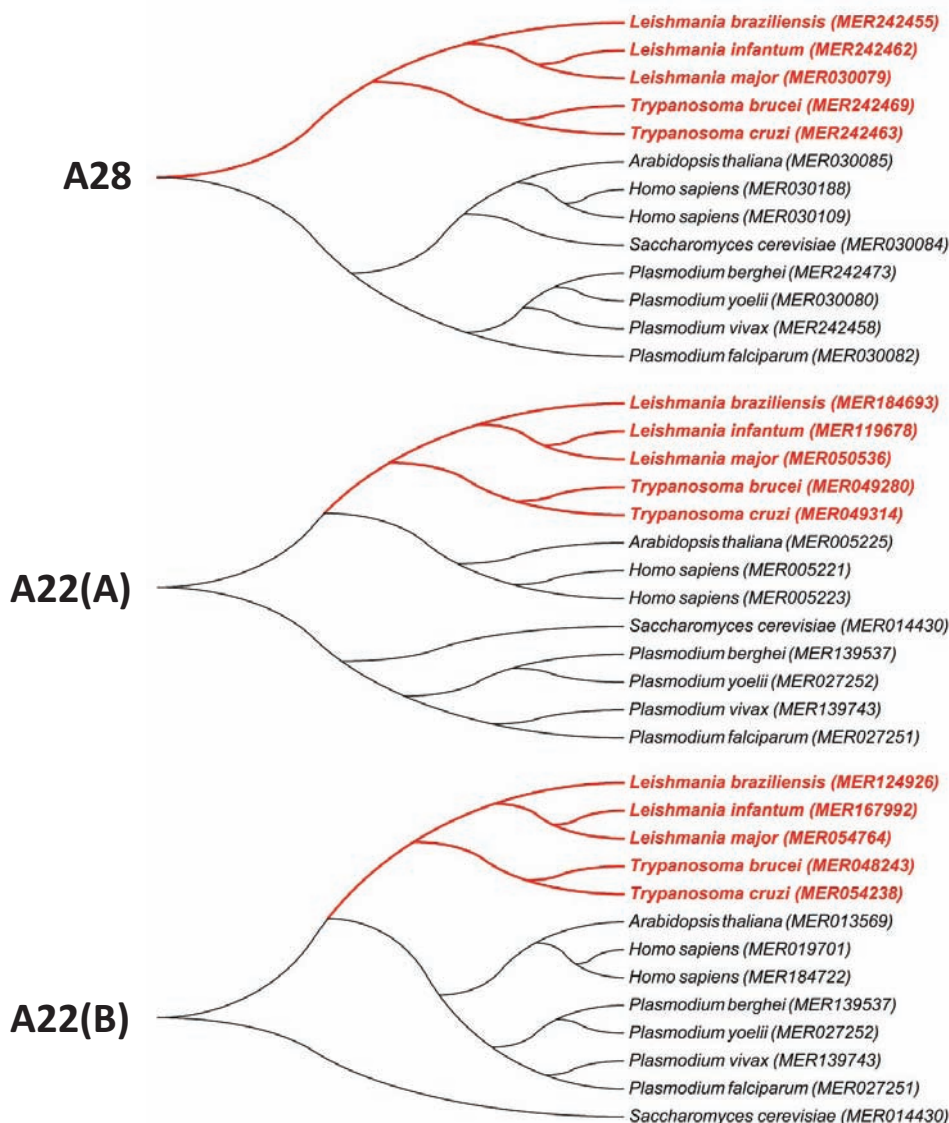
Aspartic peptidase family	Archaea	Bacteria	Eukarya				Virus
			Protista	Fungi	Animalia	Plantae	
<b>Clan AA</b>							
A1	-	11	240	853	899	888	-
A2	-	1	-	28	676	43	233
A3	-	-	-	-	-	4	31
A9	-	-	-	-	-	-	10
A11	-	-	2	240	139	1203	2
A28	-	-	39	71	71	15	-
A32	-	355	-	-	-	-	-
A33	-	-	1	-	17	9	-
<b>Clan AD</b>							
A22	66	-	54	53	394	97	-
A24	158	1188	-	-	1	1	-

Source: MEROPS - The Peptidase Database (Rawlings *et al.*, 2012), release 9.6; URL: <http://merops.sanger.ac.uk/>

growth, viability, ultrastructure and infectivity [58-63]. These approaches unequivocally illustrate the presence and relevance of this enzymatic class in the Trypanosomatidae family, and point out to the necessity to further characterize these enzymes. In the sections below, we will describe the data on the literature regarding the inhibition of aspartic peptidases produced by human pathogenic trypanosomatids, especially *Leishmania* spp. and *T. cruzi*, which highlight the possibility of an alternative target for chemotherapy.

#### 4. ASPARTIC PROTEOLYTIC INHIBITORS AS PROSPECTIVE CHEMOTHERAPEUTIC AGENTS

There are a number of comprehensive reviews on the applicability of proteolytic inhibitors as chemotherapeutic agents [64-81]. Here, we will shortly illustrate this potentiality, focusing on aspartic peptidase inhibitors. Several proteolytic inhibitors have already been used in the clinic with considerable success to treat hypertension, coagulation disorders, cancer and diabetes. They include angiotensin-



**Fig. (7).** Phylogenetic relationship aspartic peptidase members of the A28 and A22 families from trypanosomatids. Peptidase sequences obtained from the MEROPS database release 9.6 [19] were aligned with the program ClustalW version 2.1 [122]; phylogram was constructed with the software MEGA version 5 [123] after 1,000 bootstraps with the neighbor-joining algorithm [124] (A) and (B) denote different sub-families.

converting enzyme (ACE) inhibitors for treating high blood pressure, thrombin inhibitors for treating stroke, and an elastase inhibitor for treating systemic inflammatory response syndrome (SIRS) [72]. The example of greater impact and success in treatment of an infectious disease with proteolytic inhibitors is the highly active antiretroviral therapy (HAART) used to treat the acquired immunodeficiency syndrome (AIDS) (Fig. 6). The HAART has led to a marked improvement in the life expectancy of AIDS sufferers by the fall of HIV viremia and by restoring the immune responses with an increase in the number of CD4<sup>+</sup> T lymphocytes and with an effective stimulation in the survival and activation of neutrophils, monocytes, endothelial and dendritic cells. All these beneficial properties of HAART culminated in a drastic reduction of opportunistic infections [82-88]. This reduction seems to be based not only on the immune system restoration, but also on the direct inhibition of aspartic peptidases

produced by opportunistic pathogens, as demonstrated in some bacteria, fungi and protozoa [62, 63, 89-93].

The difficulty in treating parasitic diseases is partly due to the complexity of biological organisms responsible by these pathologies. Thus, there are several chemotherapeutic approaches being developed [94-95], including the use of proteolytic inhibitors to treat malaria, leishmaniasis and trypanosomiasis. For instance, the *Plasmodium* parasite, the causative agent of malaria, has proteolytic enzymes that play key roles in hemoglobin hydrolysis and this process appears to involve multiple catalytic classes of peptidases, including cysteine, metallo and aspartic peptidases. Among such enzymes, PMs and, especially, falcipains (cysteine peptidases) are highly promising antimalarial drug targets [96]. Two HIV peptidase inhibitors (HIV PIs), saquinavir and ritonavir, have been established as antimalarials in clinical use in combina-



tion with chloroquine and mefloquine [97]. Looking for an example in fungi, where the effectiveness of HAART has been more extensively explored, *Candida albicans* can be selected as a prototypal microorganism [89-93, 98-100]. *C. albicans* is part of the normal human respiratory, genital and gastrointestinal tracts flora and the major cause of opportunistic fungal infections in immunocompromised people. The secreted aspartic peptidases (Saps) are recognized as the main virulence factor of *Candida* and they belong to the same superfamily of HIV aspartic peptidase. Thus, studies have been conducted and confirmed the effect of HIV PIs on Sap activity, fungal proliferation, morphogenesis, adhesion to mammalian and experimental candidiasis infection as well as synergistic drug properties with classical antifungals [101]. The main example of beneficial effects of HIV PIs on bacteria was demonstrated against *Mycobacterium* spp. Studies have demonstrated a decline in the tuberculosis rate coincident with the introduction of HAART [102-104]. Kabbesh and colleagues [105] showed that ritonavir was able to significantly diminish the synthesis of cell wall lipids, suggesting a loss in the function of this fundamental mycobacterial structure. Collectively, these published reports exemplify the wide range of action of HIV PIs against phylogenetic distinct classes of microorganisms.

## 5. ANTI-TRYPANOSOMATID PROPERTIES OF ASPARTIC PEPTIDASE INHIBITORS

### 5.1. *Leishmania*

Peptidases have been extensively studied in *Leishmania*, and a simple analysis on the number of representative genes from each proteolytic class indicates a clear prevalence of metallopeptidases, followed by cysteine- and serine-type peptidases (Fig. 3). This scenario is reinforced by the extensive reports on *Leishmania* cysteine peptidases CPA, CPB and CPC, and the metallopeptidase GP63, which accounts for about 1% of the organism's total protein content [for a comprehensive review, see 67]. There is a general lack of knowledge about aspartic peptidases in *Leishmania*. Currently, there are only few studies describing aspartic peptidase activities in soluble fractions of crude *Leishmania* extracts, by means of selective substrates and inhibitors (Fig. 6) to this enzymatic class [58, 60, 62, 63, 106]. *L. amazonensis* soluble crude extract presents an acidic hydrolytic activity able to degrade the renin synthetic substrate NCbz-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser- $\beta$ -naphthylamide [58], the HIV-1 aspartic peptidase substrate Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg [62] and the cathepsin D substrate 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-amide [106], these activities were inhibited by pepstatin A and HIV PIs (Fig. 6) [58, 62]. Also, *L. mexicana* soluble crude extracts were shown to degrade the substrate benzoyl-Arg-Gly-Phe-Phe-Leu-4-methoxy- $\beta$ -naphthylamide optimally at pH 3.8. Although the substrate degradation was not fully inhibited by pepstatin A (only 45% of inhibition), diazo-acetyl-norleucinemethylester (DAN), another selective aspartic peptidase inhibitor (Fig. 6), virtually abolished the enzymatic activity. Peptidase inhibitors selective for other peptidase classes (E-64, leupeptin and 1,10-phenanthroline) presented only marginal effects on the hydrolysis [60]. Envisaging a

possible exploitation of aspartic peptidases as a target for chemotherapy, the authors also demonstrated the anti-proliferative effect of DAN on promastigotes, showing a 50% lethal dose of 22  $\mu$ M after 72 h of *in vitro* cultivation [60]. Moreover, DAN induced significant alterations in the shape of promastigotes of *L. mexicana*, from a long slender form to a spherical one with at least two nuclei per parasite suggesting the blockage of cell division [60]. It was also demonstrated that the aspartic peptidase activity is down regulated during the *L. amazonensis* promastigote into amastigote differentiation *in vitro* [58].

Although aspartic peptidases were never purified and fully characterized in *Leishmania*, several studies have been conducted in order to test the effects of HIV PIs on these parasites. These studies were driven by the drastic reduction in the incidence, morbidity and mortality of AIDS co-infections after the introduction of HIV PIs in the antiretroviral therapy. These inhibitors were able to promote a series of damaging effects on parasite proliferation and ultrastructure, as well as a reduced ability of *Leishmania* to infect and survive within host cell macrophages [59-63, 106]. In this sense, Savoia and colleagues [59] were the first to describe a dose-dependent effect of HIV PIs on two *Leishmania* species. The 50% lethal dose (LD<sub>50</sub>) after incubation of *L. major* for 24 h with indinavir and saquinavir was shown to be 8.3  $\mu$ M and 7.0  $\mu$ M, respectively. The inhibitory effect was more protuberant for this species, which causes cutaneous leishmaniasis, than for *L. infantum* promastigotes, a causative agent of visceral leishmaniasis. In the latter, the highest concentration tested (50  $\mu$ M) did not achieve an inhibition of 50% [59]. The HIV PIs present an irreversible effect, since parasites do not resume growth when subcultured into fresh medium [59].

Some years later, Trudel and colleagues [61] reported that ritonavir, saquinavir and nelfinavir presented no inhibitory effect on the growth of *L. infantum* promastigotes. Almost at the same time, our research group reported the anti-promastigote activity of lopinavir, nelfinavir and amprenavir against *L. amazonensis* promastigotes, while saquinavir and indinavir presented only negligible inhibition [62]. Following this publication, Valdivieso and colleagues [63] reinforced the data presented by Savoia and colleagues [59], showing the anti-promastigote activity of nelfinavir and saquinavir against a panel of *L. infantum* isolates [63]. They also showed that *Leishmania* species associated with cutaneous manifestations present values of IC<sub>50</sub> for nelfinavir and saquinavir slightly lower than those of species associated with visceral manifestations, as previously reported by Savoia and colleagues [59, 63]. There seems to be a lack of consensus on the literature about the susceptibility of different *Leishmania* species to the available HIV PIs. It is yet unclear if these discrepancies are indeed due to the wide genetic variability among *Leishmania* strains, isolates and species, or if it is due to methodological and reagent differences. This prompted our research group to challenge *L. amazonensis*, *L. braziliensis*, *L. donovani*, *L. major* and *L. infantum* with nelfinavir and saquinavir for 72 h in order to compare the results under the same standardized conditions [106]. Saquinavir was capable of statistically inhibiting only *L. donovani* growth [106], while nelfinavir inhibited in more than 90% all the species tests, except for *L. major*, which

presented an inhibition of 50%. A systematic review on *Leishmania* inhibition by HIV PIs is a difficult task due to discrepancies in methodological design, data analysis and representation, strains and species assessed, and reagents employed. Nevertheless, in (Table 4), we tried to summarize the available information.

Although there is some discrepancy on the susceptibility of *Leishmania* promastigotes to certain HIV PIs, a careful analysis of the data depicted in (Table 4) clearly indicates the anti-proliferative action of these inhibitors against promastigote forms. The next line of evidence on the potentiality of HIV PIs for leishmaniasis chemotherapy was shown by the ability of these inhibitors to impair parasite development in macrophages, which was published almost simultaneously by Trudel and colleagues [61] and our research group [62]. The work of Trudel and colleagues [61] demonstrated that, although under the conditions employed, nelfinavir, ritonavir and saquinavir did not exert an inhibitory action on promastigotes, these inhibitors exerted pronounced effects against the intracellular parasites in two *in vitro* infection cell systems: phorbolmyristate acetate-differentiated THP-1 macrophages and human primary monocyte-derived macrophages (MDM). Importantly, the efficacy of HIV PIs to reduce the intracellular growth of *Leishmania* parasites is also observed in MDMs-coinfected with HIV-1 [61]. Also, a field isolate of *Leishmania donovani* resistant to sodium stibogluconate, one of the drugs most commonly used to treat leishmaniasis, is equally susceptible to the tested PIs compared with a sensitive strain, thus suggesting that resistance to sodium stibogluconate does not result in cross-resistance to HIV PIs [61]. Our research group demonstrated that the HIV PIs can interfere in the early steps of parasite infection in macrophages, since the inhibitors were added exclusively to *Leishmania* promastigotes and that the interaction process was stopped with only 1 hour. As expected, the treatment of previously infected macrophages with HIV PIs notably reduced the association indexes, in a dose-dependent manner [62]. It is interesting to note that the HIV PIs efficacy is higher for amastigotes inside macrophages than for extracellular amastigotes or promastigotes [61-63]. This could be explained by a combination of factors: a direct anti-amastigote activity together with a modulation of the killing capability of the macrophages and a concentration of the drugs inside the macrophages.

The effectiveness of HIV PIs in treating parasitic infections may be associated to their capacity to modulate or block the cell proteasome or to promote apoptosis [78]. Alternatively, it could act directly on aspartic peptidases produced by protozoa. Our research group was the first to demonstrate that the HIV PIs are capable of inhibiting, in a dose-dependent manner, the degradation of a HIV-1 peptidase substrate at acidic pH by *L. amazonensis* [62]. This was the first line of evidence that the intracellular target of the HIV PIs in *Leishmania* could be an aspartic peptidase. It should be pointed out that the HIV PIs were designed to fit viral peptidase and may thus have a lower affinity for *Leishmania* aspartic peptidase. As a matter of fact, the HIV PIs belongs to family A2, clan AA, while the identified aspartic peptidases in *Leishmania* genome belongs to family A28 and A22, clans AA and AD, respectively (Table 2, Fig. 4). Although the viral and leishmanial peptidases belong to clan AA, similarity searches using HIV aspartic peptidase

AA, similarity searches using HIV aspartic peptidase sequences against the entire set of annotated proteins encoded in *Leishmania* genomes reveal no statistically significant hit (data not shown). However, Perteguer and collaborators [108] have recently isolated a full-length cDNA encoding a 49-kDa protein from *L. major*, which exhibited significant deduced amino acid sequence homology with the annotated *Leishmania* sp. DNA damage-inducible (Ddi1-like) protein, as well as with the *ddi1* protein from *Saccharomyces cerevisiae*. The protein exhibited an additional fragment at the N-terminal end, homologous to the ubiquitin-like (UBL) domain of this family of proteins described in other organisms, which had not been previously reported for the *L. major* Ddi1-like protein. In addition, the cloning, expression, and functional characterization of the *L. major* recombinant Ddi1-like protein demonstrated the proteolytic activity of this protein [108]. Another piece of evidence strongly suggests that *Leishmania* aspartic peptidases are the intracellular target of the HIV PIs. A *Saccharomyces cerevisiae* knockout for *ddi-1*, an orthologous of *Leishmania* aspartic peptidase (MEROPS ID MER242455, family A28, clan AA, Table 2), was functionally complemented with the *Leishmania* orthologous, reverting the phenotype to the wild one. This phenotype reversion was also induced in the wild yeast by HIV PIs [105]. In addition, two *Leishmania* strains isolated from HIV-*Leishmania* coinfecting patients under HAART treatment exhibited lower sensibility to HIV PIs *in vitro*, as demonstrated by two independent research groups [63, 106]. It is interesting to note that the parasite isolated from a patient under treatment with HIV PIs presented considerably less aspartic peptidase activity than isolates from patients untreated or treated only with reverse transcriptase inhibitors [106]. The cultivation of this isolate in the presence of nelfinavir induced a further reduction in the aspartic peptidase activity, which suggests that these enzymes are the target of the HIV PIs and are down-regulated by the selective pressure induced by the drug [106]. Indeed, it was recently demonstrated that a leishmanial aspartic peptidase can be the intracellular target of the HIV PIs [108]. The cloned *Did-1* like protein from *L. major* that was expressed in baculovirus/insect cells readily hydrolyzed a synthetic substrate for the HIV peptidase [Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg] at acidic pH. This activity was inhibited in 70 and 95% by pepstatin A at 15 mM and DAN at 500 mM, respectively. The HIV PI nelfinavir at 20  $\mu$ M reduced the activity in 60%, while E-64 and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) presented no significant effect on the hydrolytic activity. Two other synthetic substrates specific for cathepsin D, Bz-Arg-Gly-Phe-Phe-Leu-4MbNA, and Bz-Arg-Gly-Phe-Phe-Pro-4MbNA-HCl, were hydrolyzed at lower rates and were inhibited by pepstatin A and DAN, while the inhibition by HIV PIs were not assessed [108]. A 3D model of the Ddi1 like protein from *L. major* suggests that it can accommodate bulkier substrates than those accessible to HIV-1 aspartic peptidase [108].

Although it seems reasonable to assume that the HIV PIs target *Leishmania* aspartic peptidase, the possibility of non-specific or generally toxic effects of the drugs on parasite cells should not be ruled out. In this context, electron microscopic examination of *L. amazonensis* cells exposed to

Table 4. Systematic Review of the Data Available on the Susceptibility of *Leishmania* Species and Isolates to HIV PIs.

	nelfinavir		saquinavir		Ref.
	inhibition	conc.	inhibition	conc.	
<i>L. amazonensis</i> (MHOM/BR/77/LTB016)	50% (48h)	15.12	Virtually no inhibition <sup>1</sup>		[62]
<i>L. amazonensis</i> (MHOM/BR/77/LTB016)	95% (72h)	25	5%	25	[106]
<i>L. amazonensis</i> (IFLA/BR/67/PH8)	50% <sup>3</sup>	13.36	50% <sup>3</sup>	40	[63]
<i>L. major</i> (LRC-L137)			49% (24h)	6.25	[59]
<i>L. major</i> (MHOM/SU/73/5-ASKH)	50% <sup>3</sup>	13.37	50% <sup>3</sup>	46.95	[63]
<i>L. major</i> (MHOM/IL/1980/FRIEDLIN)	50% (72h)	25	21.2% (72h)	25	[106]
<i>L. infantum</i> (MHM/TN/80/IPT1)			31% (24h)	50	[59]
<i>L. infantum</i> (MHOM/MA/67/ITMAP-263)	Virtually no inhibition <sup>2</sup>		Virtually no inhibition <sup>2</sup>		[61]
<i>L. infantum</i> (MHOM/FR/78/LEM-75)	50% <sup>3</sup>	16.46	50% <sup>3</sup>	53.97	[63]
<i>L. infantum</i> (MCAN/ES/98/LLM-724)	50% <sup>3</sup>	17.59	50% <sup>3</sup>	50.87	[63]
<i>L. infantum</i> (MCAN/VE/98/IBO-78)	50% <sup>3</sup>	14.05	50% <sup>3</sup>	55.12	[63]
<i>L. infantum</i> (MHOM/ES/95/LLM-480)	50% <sup>3</sup>	18.21	50% <sup>3</sup>	48.04	[63]
<i>L. infantum</i> (MHOM/ES/98/LLM-759) <sup>4</sup>	50% <sup>3</sup>	26.89	50% <sup>3</sup>	64.46 <sup>4</sup>	[63]
<i>L. infantum</i> (MHOM/BR/1974/PP75)	96.2% (72h)	25	0% (72h)	25	[106]
<i>L. infantum</i> (MHOM/BR/2009/ANC) <sup>4</sup>	96.6% (72h)	25	0.4% (72h)	25	[106]
<i>L. infantum</i> (MHOM/BR/2009/LCS) <sup>4</sup>	96.3% (72h)	25	0% (72h)	25	[106]
<i>L. infantum</i> (MHOM/BR/2009/VCF) <sup>4</sup>	0% (72h)	25	0% (72h)	25	[106]
<i>L. donovani</i> (MHOM/IN/80/DD8)	50% <sup>3</sup>	14.1	50% <sup>3</sup>	51.89	[63]
<i>L. donovani</i> (MHOM/ET/1967/L82)	94% (72h)	25	62% (72h)	25	[106]
<i>L. mexicana</i> (MHOM/VE/80/NR)	50% <sup>3</sup>	9.85	50% <sup>3</sup>	42.08	[63]
<i>L. mexicana</i> (MHOM/ES/2002/LLM-1162)	50% <sup>3</sup>	12.44	50% <sup>3</sup>	40.67	[63]
<i>L. mexicana</i> (MHOM/BZ/82/BEL21)	50% <sup>3</sup>	10.25	50% <sup>3</sup>	39.54	[63]
<i>L. braziliensis</i> (MHOM/BR/75/M2903)	50% <sup>3</sup>	14.6	50% <sup>3</sup>	36	[63]
<i>L. braziliensis</i> (MCAN/BR/1998/619)	95% (72h)	25	13% (72h)	25	[106]

INHIBITON – Inhibition in relation to control. The number in brackets corresponds to the time when the inhibition was assayed.

Conc. – Concentration of the inhibitor in  $\mu\text{M}$ .

<sup>1</sup> The drugs were screened from 15 to 500  $\mu\text{M}$  and the growth followed from 24 to 96 h. The inhibition observed was only marginal.

<sup>2</sup> The drugs were screened at 12.5 and 25  $\mu\text{M}$  and the growth compared to the control group after 72 h of incubation, no inhibition was observed.

<sup>3</sup> The methodology described in ref [82] was elusive in relation to the time of incubation where the  $\text{IC}_{50}$  was calculated.

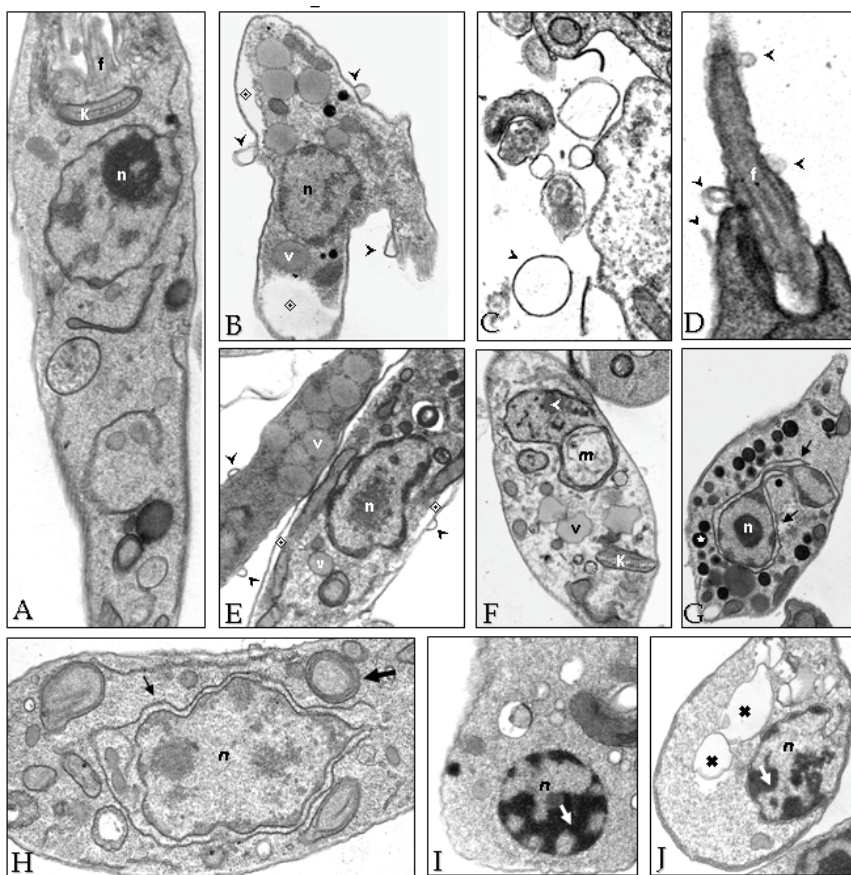
<sup>4</sup> Strains isolated from HIV/*Leishmania* co-infected patient.



nelfinavir or lopinavir revealed some peculiar alterations in vital cellular structures, such as cytoplasmic membrane and internal cellular structures, suggesting irreversible metabolic injuries that culminate in the parasite cell death [62] (Fig. 8). An interesting finding was the increase in the number of vesicles, which according to their electron-density, probably corresponds to lipid-containing compartments [62] (Fig. 8). Indeed, the HIV PIs are capable of altering the lipid composition in leishmanial cells (unpublished data). A well-known side effect of HIV PIs in humans is the lipodistrophy, which is an abdominal adiposity [109]. Also interesting to note is that some of the ultrastructural alterations observed in *L. amazonensis*, such as increase in the number of vesicles and wrapping of the nucleus by the endoplasmic reticulum, are suggestive of autophagy [62] (Fig. 8). Accordingly, it was later demonstrated that lopinavir is effective in generating oxidative stress in *Leishmania*, leading to altered physiological parameters such as increase in the sub-G1 DNA content, nuclear DNA fragmentation and loss of mitochondrial potential, which are all characteristics of

apoptosis [110]. Interestingly, HIV PIs also induced a significant increase in the expression of virulence factors (CPB and GP63) by *L. amazonensis*, when parasites were subjected to HIV PIs. One hypothesis could be that the HIV PIs are inhibiting an aspartic peptidase that should be otherwise degrading some of the GP63 and CPB peptidases. An alternative hypothesis could be that the HIV PIs are exerting stress, or some other non-specific effect (Fig. 8), on the promastigotes that leads to changes in parasite gene expression [62].

More recently, the effect of the HIV PIs indinavir and ritonavir was also tested *in vivo* using BALB/c mice infected with *L. amazonensis* in the footpad followed by oral treatment for 30 days. Antiretroviral-treated mice had a significant reduction in the footpad thickness after the third week of indinavir treatment and after the fifth week of ritonavir treatment. However, there was no reduction in the parasite load [111]. It is yet unclear why the HIV PIs have a poor efficacy in infection experiments with mice, since all data



**Fig. (8).** Ultrastructural changes observed in *L. amazonensis* after HIV PIs treatment. Parasites ( $10^8$  cells) from 48-h cultures were inoculated in fresh medium in the absence (A) or in the presence of nelfinavir (B-H) or lopinavir (I-J) at the  $IC_{50}$  concentration, and incubated for 4 h (B-E), 6 h (F-G), 8 h (H) and 24 h (I-J). Subsequently, cells were processed for transmission electron microscopy. An intense flagellar and plasma membrane shedding (black arrowheads) was seen after 4 hours of treatment with both inhibitors (B-D). Some effects were exclusive of nelfinavir, such as cytoplasm shrink (B and E,  $\diamond$ ), increase in the number of intracellular vesicles, resembling acidocalcisomes (G,  $\star$ ) and lipid inclusions (E and F,  $\nabla$ ). Both drugs induced nuclear wrapping by the endoplasmic reticulum (G and H, black arrows), mitochondrial swelling (F, white arrowheads) and myelin-like structures (H, larger arrow). In lopinavir treated cells, blocks of condensed chromatin were observed close to the nuclear envelope (I, white arrow), as well as enlarged vesicles (J,  $\star$ ). n - nucleus; k - kinetoplast; f - flagellum and m - mitochondrion. The ultrastructural alterations described for nelfinavir (B-H) were also visualized with lopinavir. Reprinted from PLoS One. 2009; 4(3): e4918. doi:10.1371/journal.pone.0004918.



pointed towards a different view, i.e., after the introduction of PIs in the antiretroviral therapy for HIV, the number of coinfecting cases reported fell sharply; PIs present an anti-promastigote activity; PIs reduce the infection in macrophages; and an aspartic peptidase seems to be the specific target of the PIs. Nevertheless, these studies strongly suggest that aspartic peptidase(s), together with HIV PIs and/or specific inhibitors to leishmanial aspartic peptidase(s), represent a promising strategy for leishmaniasis chemotherapy improvement.

## 5.2. *Trypanosoma*

Different peptidases have been extensively studied in the last decades in *T. cruzi*, with special emphasis in cysteine peptidases, represented mainly by cruzipain and the 30-kDa cathepsin B, but metallo and serine peptidases were also investigated and play critical functions for the parasite [for comprehensive reviews see 67, 112]. The group of aspartic peptidases, on the contrary, has been only recently analyzed. In this group, two aspartic peptidase activities were identified and isolated from *T. cruzi* epimastigote forms (Y strain): cruzipain-I (CZP-I) and cruzipain-II (CZP-II) [113]. The enzymes were purified by affinity chromatography through the use of the classical aspartic peptidase inhibitor, pepstatin A, coupled to agarose. Interestingly, CZP-I was isolated from cell pellets after freezing-thawing and centrifugation, followed by solubilization with the non-ionic detergent CHAPS, while CZP-II was isolated from the soluble fraction after cells lysis. The molecular mass of both peptidases was estimated to be 120-kDa by HPLC gel filtration, and the proteolytic activity of both enzymes was detected as a doublet of bands (56- and 48-kDa) by gelatin-containing sodium dodecyl sulfate polyacrylamide gel electrophoresis, which suggested that the active *T. cruzi* hydrolases are dimeric proteins composed of identical subunits of 56-60 kDa associated by bonds, similar to the vertebrate aspartic peptidases [113].

The identification of CZP-I and CZP-II as aspartic peptidases was achieved through distinct methods [113]. At first, substrate specificity studies indicated that the enzymes showed maximal proteolytic activity over the cathepsin D substrate Phe-Ala-Ala-Phe-(4-NO<sub>2</sub>)-Phe-Val-Leu-O<sub>4</sub>MP at pH 3.5-4.0, but failed to hydrolyze serine and other peptidase substrates. In addition, the proteolytic activities of the CZP-I and CZP-II fractions were strongly inhibited by pepstatin A and the aspartic active site labeling agent 1,2-epoxy-3-(phenyl-nitrophenoxy) propane (EPNP) (Fig. 6), but not by various other inhibitors of serine, metallo or cysteine peptidases. The authors emphasized that the selective inhibition by EPNP indicates that both *T. cruzi* proteolytic activities possess the dual aspartates at the active site, the signature configuration of aspartic peptidases belonging to clan AA, family A1, in which pepsin is the family-type peptidase. In this sense, it is worth mentioning that the *T. cruzi* Genome Project [114] reported only three aspartic peptidases, two of which belong to clan AD, family A22, being presenilin 1 the family-type peptidase, classically inhibited by pepstatin A. All members of this clan have transmembrane domains [19]. The third member of this group found in *T. cruzi* genome belongs to clan AA, family A28, in which the family-type peptidase is the Ddi-1 from *Saccharomyces cerevisiae* (Table 2). This protein was previously found to be the ligand for

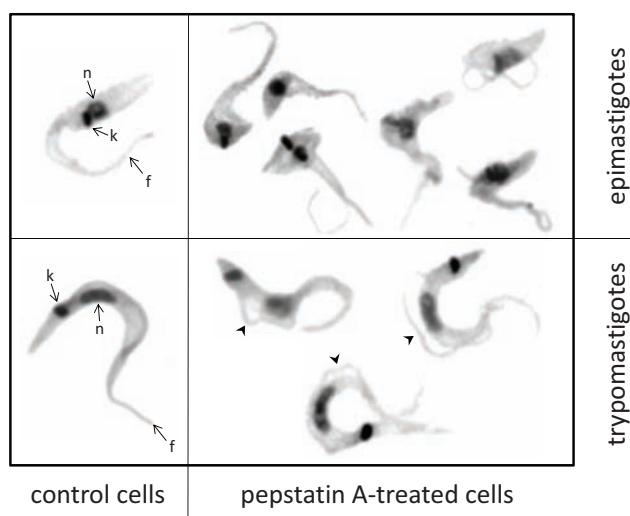
nelfinavir in *L. major*, affecting growth, proliferation and survival [107, 108]. No peptidase activity has been shown for any member of this family [19]. Interestingly, genes predicting enzymes belonging to the A1 family have not been found yet in the *T. cruzi* genome. As pointed out by Alvarez and colleagues [112], since no amino acid sequences were reported for CZP-I and CZP-II it is not possible to link these enzymes to any of the genes detected in *T. cruzi*. Nevertheless, Pinho and colleagues [113] reinforced that there are several sequences that could not be correctly identified in the *T. cruzi* genome due to difficulties in correlating homologous genes by using the current computer techniques. *T. brucei* Genome Project [114] also presented the same aspartic peptidase sequences detected for *T. cruzi* and *Leishmania* (Table 2), although no study concerning this class of proteolytic enzymes has been performed yet in African trypanosomes. Also, the efficacy of aspartic peptidase inhibitors, including HIV PIs, in *T. brucei* is a rich unexplored area, which is a point of interest of our research group.

Our group has recently begun to work with the effects of the aspartic peptidase inhibitor pepstatin A against *T. cruzi* clone Dm28c epimastigote forms [115]. Pepstatin A arrested the parasite proliferation in both dose- and time-dependent manner, resulting in significant morphological alterations, including reduction of the cell size and detachment of parts or the whole flagellum from the cell body (Fig. 9), though cell lysis was not observed. Curiously, the aspartic peptidase inhibitor induced the metacyclogenesis process, which may be connected to the stress promoted in the parasite cells. The epimastigote-to-trypomastigote differentiation was stimulated in a dose-dependent manner, but approximately 45% of the trypomastigotes had their flagellum detached from the cell body (Fig. 9). The treatment of epimastigotes with pepstatin A at the IC<sub>50</sub> value (36.2 μM) 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. As previously reported by Santos and colleagues [62], the blockage of a class of peptidase by a proteolytic inhibitor can induce an augment in the expression of distinct classes of peptidase in order to compensate its function.

Growth inhibition in the presence of the aspartic peptidase inhibitor was also observed in *T. cruzi* strains belonging to distinct phylogenetic lineages: similar levels of inhibition were obtained between clone Dm28c (DTU I) and strains CL Brener (DTU VI) and 4167 (DTU IV), while inhibition of Y strain (DTU II) and 3663 strain (DTU III) was lower in comparison to clone Dm28c [115]. These results are in accordance to the great heterogeneity of natural populations of *T. cruzi* in biological, biochemical, immunological and molecular features, which must be correlated to distinct clinical manifestations and chemotherapy response [116]. For instance, *T. cruzi* strains are able to express different amounts of peptidases, including the major cysteine peptidase cruzipain [117, 118].

The possibility of aspartic peptidase activity as the intracellular target of this inhibitor was suggested by the hydrolysis of a cathepsin D fluorogenic substrate (7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-Arg-amide) by *T. cruzi* epimastigote extract and the inhibition of its hydrolysis by pepstatin A [115]. These

results opened the possibility of exploiting aspartic peptidases as promising targets to treat Chagas' disease. As a matter of fact, our group started recently to explore the effects of HIV PIs against *T. cruzi* epimastigotes, and we observed a strong anti-proliferative effect, with  $IC_{50}$  values much lower than pepstatin A, some of them in the nanomolar range (unpublished results). Although there are no studies in the literature about the effect of aspartic peptidase inhibitors on amastigotes or trypomastigotes of *T. cruzi*, evidences show that compounds may be more effective depending on the developmental stage of the parasite. In *Leishmania*, for instance, a considerable difference in susceptibility was observed between promastigotes and amastigotes *in vitro* [61-63]. Also, leishmania-infected mice treated with HIV PIs presented a modest reduction in footpad thickness, and no reduction in parasite load [111]. In this sense, it is interesting to assess the effect of HIV PIs against the clinically relevant forms of the *T. cruzi*.



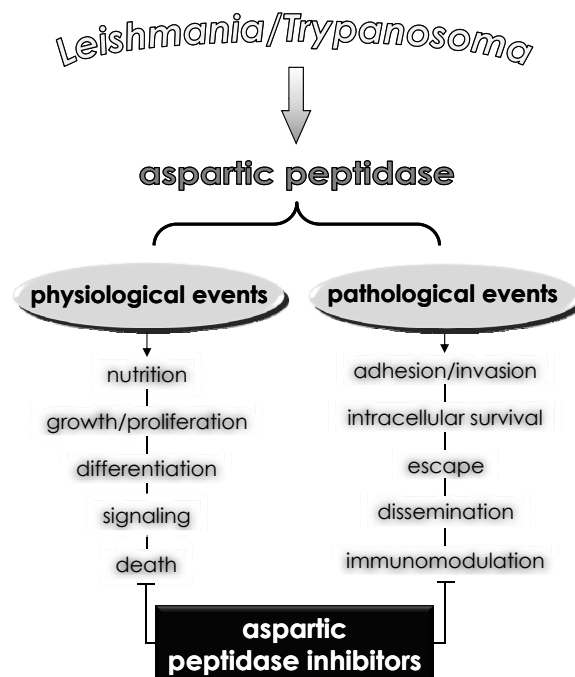
**Fig. (9).** Morphological alterations observed on both epimastigotes and trypomastigotes of *Trypanosoma cruzi* after 72 h of treatment with 50  $\mu$ M of pepstatin A. Comparing with untreated epimastigotes, pepstatin A induced the swollen in cell body and detachment of the flagellum. In trypomastigotes the most evident effect was partial and whole flagellum detachment (arrowheads). k, kinetoplast; n, nucleus; f, flagellum.

## CONCLUDING REMARKS

*Leishmania* spp. and *Trypanosoma* spp. are responsible for substantial global morbidity, mortality and economic adversity in tropical and subtropical regions, especially affecting the less developed countries. Environmental changes, drug resistance and immunosuppression contribute to the emergence and spread of these diseases. For instance, the HIV pandemic has modified the immunopathogenic, epidemiological and therapeutic aspects of these human parasitic diseases. Corroborating these outcomes, the current therapeutic arsenal against the human pathogenic trypanosomatids *T. brucei*, *T. cruzi* and *Leishmania* spp. is clearly inadequate and underscores the urgent need to develop new effective, safe and cost-effective drugs.

In view of this, a number of new strategies to obstruct trypanosomatid biological processes have emerged; one of

them is focused on peptidase inhibition. This particular class of hydrolytic enzymes cleaves peptide bonds in proteinaceous substrates, a reaction extremely important in maintaining the physiology of all living cells (Fig. 10), also peptidases are essential virulence factors for these protozoa during all stages of the infection process (Fig. 10), which make them potential targets for the development of anti-trypanosomatid drugs (Fig. 10). Supporting this view, a sharp decrease in the incidence of visceral leishmaniasis in Europe and Africa was observed following the widespread use of HAART, particularly after the introducing of aspartic peptidase inhibitors to the cocktail, further supporting the notion that HAART helps to prevent visceral leishmaniasis in individuals co-infected with *Leishmania* and HIV [118-121]. These clinical records led the researchers around the world to focus in the possibility to test aspartic peptidase inhibitors against human pathogenic trypanosomatids.



**Fig. (10).** Possible roles played by aspartic-type peptidases produced by human pathogenic trypanosomatids belonging to the *Leishmania* and *Trypanosoma* genera. Aspartic peptidases contribute to maintaining basic metabolic processes in trypanosomatid cells, which govern crucial biological events including proliferation, differentiation as well as signaling and death pathways. In addition, aspartic peptidases can also participate in different contexts of the trypanosomatid-host interface, facilitating some pathogenic events such as dissemination, adhesion, escape, nutrition and immunomodulation of the host immune responses. Consequently, aspartic peptidase inhibitors are able to block one or several of these fundamental events, reducing the ability of these trypanosomatids in causing infections.

The attenuation of parasitic infections in HIV-infected individuals might not solely have resulted from improved immunological status, but also as a result of direct inhibition of aspartic peptidases produced by parasites. Taking it in consideration, some researchers showed that *T. cruzi* and different species belonging to the *Leishmania* genus are able to produce cell-associated aspartic peptidases sensible to

classical aspartic proteolytic inhibitors (e.g., pepstatin A, DAN and EPNP) and HIV PIs (Fig. 6) used in the poliochemotherapy administered to the HIV-infected individuals. The inhibition of trypanosomatids' aspartic peptidases was capable in interfering with fundamental events of these microorganisms. In this context, several studies described the inhibitory effects of HIV PIs on (i) crucial physiological processes including loss of viability/motility, blockage of proliferation/growth, failure to maintain both morphology and cellular homeostasis, and induction of an augmentation in the level of reactive oxygen species that triggers two distinct death pathways, apoptosis and autophagy, (ii) relevant steps of trypanosomatid-host relationships such as inability to either adhere or survive inside of phagocytic cells (Fig. 10). Together, all these beneficial effects culminate in death of the microorganism and/or its inadequate ability to develop an efficient and successful infection in murine model.

Regarding to the future, the purification of aspartic peptidases produced by trypanosomatids, the more accurate knowledge of its biochemical properties and the crystallization of the tertiary structure will contribute to better understanding of the functioning of these proteolytic enzymes as well as allowing the design of more specific inhibitors. It is advisable to focus drug discovery efforts towards new mechanisms of action, in order to be successful at circumventing the problem with existing resistances, and aspartic peptidases can be a real possibility.

#### CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

#### ACKNOWLEDGEMENTS

This study was supported by grants from the Brazilian Agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ), Conselho de Ensino e Pesquisa para Graduados da Universidade Federal do Rio de Janeiro (CEPG-UFRJ) and Fundação Oswaldo Cruz (FIOCRUZ). André L.S. Santos, Marta H. Branquinho and Cláudia M. d'Avila-Levy were supported by CNPq and FAPERJ fellowships.

#### ABBREVIATIONS

ACE	=	Angiotensin-converting enzyme
AEBSF	=	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
AIDS	=	Acquired immune deficiency syndrome
BACE	=	$\beta$ -site amyloid precursor protein cleaving enzyme
CZP	=	Cruzipsin
DAN	=	Diazo-acetyl-norleucinomethylester
Ddi-1	=	DNA-damage inducible protein 1
EPNP	=	1,2-epoxy-3-(phenyl-nitrophenoxy) propane
HAP	=	Histo-aspartic peptidase
HAART	=	Highly active antiretroviral therapy

HIV	=	Human immunodeficiency virus
kDNA	=	Kinetoplast DNA
LD <sub>50</sub>	=	50% lethal dose
MDM	=	Human primary monocyte-derived macrophages
PIs	=	Peptidase inhibitors
PMs	=	Plasmepsins
SAPs	=	Secreted aspartic peptidases
SIRS	=	Systemic inflammatory response syndrome
WHO	=	World Health Organization

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