Proteinase Inhibitors: A Promising Drug Class for Treating Leishmaniasis

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Abstract: This review presents and discusses the current status and perspectives of leishmaniasis treatment, with a special focus on the use of proteinase inhibitors. The history of treatment development, the first- and second-choice modern drugs and the advantages and disadvantages of using proteinases inhibitors as leishmanicidal treatments are presented and discussed. The reports gathered herein confirm the potential usefulness of proteinases inhibitors as an alternative or complement to the current leishmaniasis treatments. They also support the hypothesis that a combined treatment with multiple proteinase inhibitors may be efficient against Leishmania infections in vertebrate hosts.

Keywords: Leishmaniasis, Leishmania, proteinases, proteinase inhibitors, treatment, virulence factors.

INTRODUCTION

Leishmaniasis is considered an endemic disease in 88 countries and is found in the Americas, Africa, Asia, Eastern Europe and Oceania [1, 2]. It is a tropical disease that primarily affects populations in poor or emerging countries. The World Health Organization estimates that the incidence of this disease, considering all clinical forms, is approximately 1.5 million new cases/year; however, this number is likely underestimated due to difficulties in identifying all cases and the occurrence of asymptomatic infections [2].

This parasitic disease is caused by species of the genus Leishmania and is acquired by a vertebrate host during the blood meal of phlebotomine sandflies. After entering the host as promastigotes, the parasites undergo morphological changes into amastigotes and become able to infect cells in the skin, mucosa or cartilage, thus causing the cutaneous form of the disease (CL). Depending on the parasite species and strain, the amastigotes may colonize cells in internal organs, including the liver, spleen and bone marrow, to cause the visceral form of the disease (VL) [3]. In contrast to extracellular promastigotes that possess a visible flagellum, amastigotes are intracellular round-shaped forms that preferentially inhabit cells of the mononuclear phagocyte system. This last morphological stage of the parasite is the preferred target for the development of novel chemotherapies because it is the parasite stage that inhabits vertebrate hosts.

However, despite the high incidence rate and the commitment of researchers working to develop new drugs for leishmaniasis treatment, little success has been achieved. The current drugs used in the treatment of leishmaniasis have limitations regarding their use, such as high cost, difficulty of administration, toxicity or the occasional development of resistant parasite strains, which represent obstacles to successful therapies [4]. For this reason, there is a dire need to identify novel and specific drugs to treat Leishmania infections.

HISTORY AND CURRENT STATUS OF LEISHMANIASIS TREATMENT WITH ANTIMONIALS

Despite the length of time that the Leishmania parasites have been known (the first report dates back to 1885 by David D. Cunningham) [5], leishmaniasis remains a major cause of suffering for many millions of people in the tropical and subtropical regions of the world. This situation is due to the absence of effective treatment options, inadequate control practices and little to none interest of the major pharmaceutical industries to research for novel treatment alternatives. This lack of interest characterizes leishmaniasis as a tropical neglected disease.

The current treatment for leishmaniasis is based on that proposed by Gaspar Vianna in 1912, who was able to effectively treat cutaneous leishmaniasis patients by intravenously injecting emetic tartar [6]. His approach was inspired by the then promising microbicidal effects that antimonials presented against other trypanosomatid species [7].

In 1915, Vianna’s treatment has also been shown to be effective against visceral leishmaniasis, as reported by Cristiana and Caronia in Italy [8] and by Rogers in India [9]. However, the treatment has disadvantages, as the drug is highly toxic to the patients and is very unstable in the tropical climate. Antimony has even been reported to have no beneficial effect in other studies [10, 11].

This controversy led to further studies and, in 1920, to the development of a new antimonial compound to treat kala-azar: urea stibamine by Upendranath Brahmachari [12]. The next developments in leishmaniasis treatment were achieved only decades later with the advent of the pentavalent antimonials: sodium antimonyl (V) gluconate in 1937.
these antimonials, has been described as a pivotal step for the antileishmanial activity of these compounds [26].

The additional microbicidal effects of the antimonial compounds include the following: the induction of apoptosis in amastigotes by Sb$^{5+}$, as observed by DNA fragmentation and exposure of phosphatidylserines on the outer surface of plasma membrane in parasites exposed to antimonials [27]; the inhibition of topoisomerases [28]; the formation of complexes with ribonucleosides [29]; and, interference in the translocation of preformed purines [30].

Regarding the current treatment posology, the WHO recommends a dose of Sb$^{5+}$ of 10 to 20 mg/kg/day intramuscularly or intravenously with a maximum total daily dose of 810 mg of Sb$^{5+}$. The treatments generally continue for 20 days but may be reduced to a minimum of two weeks if the anticipated parasitological cure is observed. In cases in which systemic use of Sb$^{5+}$ is contraindicated, 0.2 to 1 ml of the antimonial solution may be administered directly into the lesions [11, 31, 32].

The use of antimony is characterized by a broad spectrum of mild to moderate adverse effects. The most common of these adverse effects are myalgia, arthralgia, nausea, vomiting, fever, headache, abdominal pain, pain at the site of application and edema. Nevertheless, these side effects are seldom considered severe enough to necessitate treatment discontinuation. The most hazardous side effect associated with antimonials is undoubtedly cardiotoxicity. This effect is markedly increased when Sb$^{3+}$ is used, but it may also occur with high doses of Sb$^{5+}$. The cardiotoxicity associated with antimonials is characterized by several changes in the cardiovascular system, particularly altered ventricular repolarization [32, 33].

SECOND-CHOICE DRUGS FOR LEISHMANIASIS TREATMENT

In cases where there are contraindications for antimonial-based treatments, when patients present intolerance or due to emergence of resistant parasite strains, the use another set of drugs is recommended, as Amphotericin B, Pentamidine, Miltefosine or Paromomycin. Therefore, those are classified as second-choice drugs [15].

Amphotericin B

Amphotericin B deoxycholate is a polyene antibiotic obtained from Streptomyces nodosus, with well-known antifungal activity and reported to be effective against Leishmania promastigotes and amastigotes both in vitro and in vivo [34]. This drug has been applied for the treatment of VL in India and Brazil for many years and has proven to be an effective yet difficult treatment. Amphotericin B has also been shown to be effective against the mucosal form of leishmaniasis, in which relapses are common [35-38]. Attempts to reduce the side effects of amphotericin B led to the development of lipid formulations of this drug that encapsulate it in micelles. The lipid particles are quickly removed from patient circulation by mononuclear phagocytes that then deliver large quantities of the drug inside the infected cells, thus enhancing its antiparasitic effects.
Currently, the following three lipid formulations are available: liposomal amphotericin B (AmBisome; Nexstar, USA); amphotericin B lipid complex (Abelect, ABLC; Liposome Co., USA); and amphotericin B colloidal dispersion (Amphocil, Amphotec; Sequus, USA) [35, 37, 39]. These formulations are similar to amphotericin B deoxycholate in their efficacy but are significantly less toxic. The liposomal formulation of amphotericin B is used for the treatment of VL only in Europe due to its very high cost, which precludes its use in developing countries [40, 41].

The mechanism of action of amphotericin B is due to its reaction with sterols that contain a methyl substitution in C-24 (episterol and ergosterol) in the parasite cell membrane, thus forming pores that alter the ionic balance, cell permeability and, eventually, cause cell death. However, this drug can also bind to cholesterol molecules present in the cell membrane of host cells, thus causing toxic side effects in the patients [17, 35, 40, 42]. This drug is highly effective against Leishmania; in assays with hamsters or monkeys infected with L. (L.) donovani, it was described to be 400 times more potent than pentavalent antimonials against the parasite.

In Brazil, the recommended dose of amphotericin B for VL or CL treatment is 1 mg/kg/day on alternate days for 20 days. The recommended total dose ranges from 1.0-1.5 g for CL treatment and 2.5-3.0 g for VL [16, 40, 43].

Despite its high effectiveness, amphotericin B is used as a second-choice drug due to its serious adverse effects and many treatment drawbacks, including the need for parenteral administration, long-term therapy and constant clinical monitoring. Thus, it is usually only used in cases where treatment with pentavalent antimonials did not produce an adequate response, however it is considered the first-choice drug for the treatment of pregnant women [17, 35, 37, 40, 42].

**Pentamidine**

Pentamidine is an aromatic diamine used for the treatment of patients who are unresponsive to therapy with antimonials. This drug is also applied in the treatment of incipient cases of Rhodesian or Gambia trypanosomiasis. It was first introduced as an antileishmanial agent in 1952 and has been used in the treatment of various clinical forms of leishmaniasis. However, its high toxicity and low effectiveness compared with other treatment options led to a suspension of its use in several countries [44-47].

Pentamidine’s mechanism of action appears to be related to its ability to bind kinetoplast DNA in the parasites and thus affect their survival. However, this hypothesis requires further investigation, and other potential effects of pentamidine on the parasites must be addressed [41, 48, 49].

Pentamidine isothionate is preferably administered by intravenous infusion or, alternatively, intramuscularly, as it is readily absorbed and exits the circulation rapidly. The recommended dose is 7 mg/kg (corresponding to 4 mg of pentamidine base) in 48-hour intervals. Alternatively, a dose of 2 mg/kg of pentamidine base may be administered in seven injections. The total dose of pentamidine base in the treatment should not exceed 2 g [16, 43].

The most common adverse effects related to the use of pentamidine isothionate are pain and sterile abscesses at the injection site, nausea, vomiting, dizziness, malaise, myalgia, arthralgia, headache, hypotension, syncope, cytolysis of pancreatic beta cells, hypoglycemia and hyperglycemia. In extreme cases, cardiotoxicity may occur, leading to fatal arrhythmia. Another major toxic effect of pentamidine is the development of insulin-dependent diabetes in treated patients; this effect has an incidence rate of 12.5% in cases in which the total dose of treatment nears 1 g [17, 35, 40, 50].

**Miltefosine**

Miltefosine, a hexadecylphosphocholine, was originally developed as an oral antineoplastic agent (for cutaneous cancer treatment). After a series of clinical studies between 1997 and 2000, it was approved under the commercial name ImpavidoTM, becoming, in some countries, the first available oral treatment for leishmaniasis [51].

Its mechanism of action against the Leishmania parasites appears to be via the modulation of cell surface receptors that affect many relevant cell processes, including calcium homeostasis, ether-lipid remodeling mechanisms, the synthesis of phosphatidylcholine, signal transduction, inositol metabolism, phospholipase activation and protein kinase C, as well as other mitogenic and apoptotic pathways. Miltefosine also increases macrophage cytotoxicity by increasing oxidative stress and stimulating cellular glucose consumption (by the production of reactive oxygen species such as H2O2 and superoxide O2), eventually leading to the death of the parasites inhabiting these cells [52-57].

Miltefosine has been used at doses of 2-2.5 mg/kg/day or 50 mg twice a day for 28 days. It is noteworthy that the efficacy of this drug in the treatment of CL in the New World is limited [16, 43, 58].

The most common adverse effects observed with miltefosine are related to the gastrointestinal tract and include diarrhea and vomiting. These effects occur in more than 30% of the treated patients, and its use is contraindicated during pregnancy because it has known teratogenic effects. Severe symptoms may occur when doses as high as 200 mg per day are used [35, 59, 60].

**Paromomycin**

Paromomycin, also known as aminosidine, is the only aminoglycoside with clinically important antileishmanial activity; both the visceral and cutaneous forms can be treated with this antibiotic. Due to its poor oral absorption, a parenteral formulation for VL treatment and a topical formulation for CL treatment have been developed [61-63]. Paromomycin has been tested against VL at dose of 15-20 mg/kg of paromomycin sulfate for 21 days [16]. The following three topical formulations have been used for cutaneous leishmaniasis: 15% paromomycin with 12% methylbenzethonium chloride; 15% paromomycin with 10% urea; and 15% paromomycin with 0.5% gentamicin. All of these formulations are administered twice a day for up to 20 days. These formulations have shown varying results depending on the species of Leishmania involved [43]. A notable advantage of paromomycin is observed when it is applied in combination with antimonials; in this case, paromomycin aids in reducing the therapy duration from 30 days to 17-21 days.
The exact mechanism of action of paromomycin requires further elucidation, but it has been reported to inhibit protein synthesis in protozoans by binding to the 30S ribosomal subunit and causing an accumulation of abnormal initiation complexes [64].

Similar to other aminoglycosides, paromomycin has several adverse effects, including ototoxicity, nephrotoxicity, eighth cranial nerve disease and liver function abnormalities [62].

**Azoles**

The many azole compounds have been widely used as oral antifungal agents as they are well tolerated by the patients and efficient to treat these infections [65, 66]. They have been suggested for clinical treatment of leishmaniasis, as these drugs have shown antileishmanial activity in vitro and in vivo, by inhibiting ergosterol biosynthesis in the parasites and thereby affecting their cell membrane.

Fluconazole has been reported to show promising results in the treatment of cutaneous or visceral leishmaniasis caused by parasites of both subgenera [67-69], although its efficiency, applicability or required dosage are still in debate [70-73].

Itraconazole presents similar contradictory data in the literature: there are reports of clinical cases where patients have been successfully treated for cutaneous leishmaniasis with this azole [74, 75]; but, in a larger clinical trial, it was noted that the cure rates of patients with cutaneous leishmaniasis were similar between the placebo group and the group treated with itraconazole [76].

Ketoconazole has also been used in studies with small patient numbers infected with species from both the Old and New Worlds and presented an acceptable cure rate [77-79]. However, these results still require confirmation by further larger studies.

**PROTEINASES AS POTENTIAL TARGETS FOR NOVEL LEISHMANIASIS TREATMENTS**

Proteolysis is a common mechanism of activation or inactivation of enzymes involved in an array of biological processes, such as digestion, blood clotting, cell differentiation and apoptosis [80].

Peptide bond hydrolysis can occur at the amino or carboxyl-terminal position of a polypeptide chain (exopeptidase activity) or within the polypeptide (endopeptidase or proteinase activity). The proteinases are classified based on the amino acid residues present in their catalytic site. The most common proteinase classes are serine, cysteine, aspartyl, metallo, threonine and glutamine proteinases. Among these, four classes have already been described in Leishmania parasites: serine, cysteine, aspartyl and metallo proteinases [81, 82].

Serine proteinases (SPs) contain a characteristic catalytic triad (histidine, serine and aspartic acid) in their active site. The hydrolytic activity of this triad occurs when the histidine and the aspartic residues interact with a serine residue and deprotonate a hydroxyl group. The enzyme then performs a nucleophilic attack on a carbonyl carbon of the substrate and hydrolyzes it [83].

Cysteine proteinases (CPs) have a hydrolytic mechanism similar to that of SPs; however, the active nucleophile radical is the thiol group of a cysteine residue rather than a hydroxyl group of a serine residue [83].

Aspartyl proteinases contain two aspartic acid residues in their active site that can act similar to an acid/base mechanism. In these enzymes, a water molecule coordinated between the two aspartic residues is activated by deprotonation and then attacks a carbonyl group in the substrate [83].

Metalloproteinases (MPs) contain a coordinated metal atom in their structure, usually zinc, which stabilizes the oxyanion hole. In many MPs, such as thermolysin and matrix MPs, two or three histidine residues and an acidic side chain perform the coordination of the metal ion. A water molecule is deprotonated by the coordinated metal ion and serves as the agent of hydrolytic activity against the substrate [83].

Despite their distinct and specific mechanisms of action, proteinases from these classes have been reported, in different studies and to variable degrees, to be virulence factors with relevant activity during the processes of Leishmania infection establishment and evolution in the vertebrate hosts.

**Proteinases of Leishmania spp. as Virulence Factors and Targets for Novel Treatments**

In the mammalian host cell, especially those of the mononuclear-phagocytic system, Leishmania parasites inhabit the parasitophorous vacuole (PV), which is originated by the fusion of a parasite-containing phagosome with other organelles, such as lysosomes and endosomes, characterized by an acidic environment (pH 4.7-5.2) with a great diversity of macromolecules [84, 85].

This potentially hostile environment is also the site where the metacyclic promastigotes that entered the mammal host differentiate into amastigotes, and must be able to adapt to the PV conditions [86]. In addition to this pivotal adaptation to the PV, the amastigotes must also be able to avoid or subvert the host’s immune responses to further infect other cells.

It is in such conditions that the proteinases of Leishmania develop their activities and participate in nutrients acquisition, metabolic turnover and, as previously mentioned, host-parasite interactions.

Our group has previously extensively reviewed the data currently available in the literature regarding the roles of proteinases as virulence factors in Leishmania infections [82].

CPs are currently the protein class with the most reports of activity as virulence factors in Leishmania and are prevalent in species that belong to the L. (L.) mexicana complex [86]. Of all of the distinct CPs of these parasites, three papain-like CPs have been most thoroughly analyzed (CPA, CBP and CPC) [87].

CP activity is higher in L. (L.) amazonensis amastigotes, the evolutive form that infects mammals, than in promastigotes [88], which inhabit the gut of sandflies, and there is a correlation between the levels of CP expression and the infectivity of the parasite [89]. Thus, these proteinases have potential to influence the outcome of the infection.
Studies of CP gene suppression in *Leishmania* spp. have further demonstrated the important role of these proteinases. The suppression of CP expression diminishes the infectivity of *Leishmania (Leishmania) infantum* in hamsters [90] and that of *Leishmania (Leishmania) chagasi* in human cell cultures [91]. In addition, suppressing the multiple copies of the CPB genes in *L. (L.) mexicana* reduces the capacity of the parasites to infect and induce lesions in the hosts [92, 93].

CPB has been reported to be the most relevant CP for parasites of the *L. (L.) mexicana* complex, as it exerts an extensive array of effects on the vertebrate host. This enzyme promotes interleukin (IL)-4 expression [94], inhibit IL-12 expression [95], affect the transcription factors STAT-1, AP-1 and NF-κB, impair nitric oxide (NO) production [96] and cleave major histocompatibility complex (MHC) class I proteins [97].

A specific portion of CPB, the COOH-terminal extension, is not observed in other CPs and has been implicated in the infection process in the vertebrate host. This domain of CPB influences the production of cytokines and NO by the host and affects the capacity of parasites to survive inside macrophages [98-100].

Both CPA and CPC have been reported to play roles in the parasite-host interaction; however, these enzymes are less relevant to infection than CPB. Their suppression leads to fewer significant effects. *L. (L.) mexicana* parasites with a suppressed CPC gene are more susceptible to killing by host cells [101, 102], and *L. (L.) infantum* parasites with a suppressed CPA gene are less able to infect mammalian hosts [103].

Regarding MPs, the major surface protein (MSP or gp63) is abundantly expressed on the surface of *Leishmania* parasites [104] and has known roles as a virulence factor. GP63 is required for the promastigotes to survive complement-mediated lysis in the mammalian host [105, 106], modulates certain cytokine responses in the host [107], and it affects the proliferation of natural killer cells during the infection [108]. This MP also affects transcription factors and signal transduction cascades and can cleave c-Jun (the central component of the transcription complex AP-1) [109] and NF-κB [110], and it also activates tyrosine phosphatases in macrophages [108]. Interestingly, GP63 also affects the host immune responses by cleaving CD4 glycoprotein, as observed in assays with human T cells [111].

Some studies have also identified roles of SPs as virulence factors. In attenuated strains of *L. (L.) donovani*, the surface SP levels are decreased, and the presence of a 115 kDa SP affects the ability of parasites to infect their hosts [112]. Moreover, the expression of oligopeptidase B appears necessary for *Leishmania* parasites to remain undetected in macrophages during infection [113].

As the above data indicate, proteinases are highly relevant factors for many species of *Leishmania* and participate in pivotal processes of the parasite life cycle. Understanding the distinct mechanisms of proteinase action and their importance in the biology of the parasites are necessary to adequately define the potential of proteinase inhibitors for infection treatment.

### Effects of Proteinase Inhibitors on *Leishmania* Parasites in Culture

Previous studies have reported the inhibitory effects of different classes of proteinases on the survival and/or proliferation of *Leishmania* parasites. These studies often describe the use of viral proteinase inhibitors, especially those targeted to HIV proteinases [114-117].

Certain HIV aspartyl proteinase inhibitors, including Ac-Leu-Val-Phenylalanine, saquinavir mesylate and nelfinavir, impair *Leishmania* cell division. In addition, these drugs have been shown to decrease the number of monocytes co-infected by HIV/*Leishmania* in culture, in a dose-dependent fashion [118]. Together, these reports indicate the potential of using these drugs for leishmaniasis treatment and underline the need to develop drug design studies to increase their affinity for the *Leishmania* proteinases.

Another promising front for the search of new antileishmanial drugs is research into natural compounds obtained from plants or other organisms. Interestingly, the mechanism of action of some of these compounds is through proteinase inhibition.

The bio avonoid fukugetin is a compound isolated from the fruits of *Garcinia brasiliensis* by ethyl-acetate extraction, and it has been shown to inhibit the activity of *L. (L.) amazonensis* cysteine and serine proteinases. However, this compound showed no activity against promastigotes or amastigotes in vitro [119].

A Kunitz-type serine proteinase inhibitor obtained from a sea anemone, named ShPI-I, was able to affect SP activity in promastigotes of *L. (L.) amazonensis* and also, to reduce parasites viability in culture, inducing morphological alterations to the cells [120]. This inhibitor’s effects on parasites viability and morphology were more pronounced than those of classic SP inhibitors (N-tosyl-L-phenylalanine chloromethyl ketone and benzamidine) at the same time point but in lower concentration, suggesting an effective antileishmanial activity.

In addition, chemically synthesized compounds with proteinase-inhibiting properties that have not been previously studied for the treatment of other diseases have been analyzed with regard to their effects on *Leishmania* parasites. MP-inhibiting synthetic compounds have been selected through *in silico* analysis from databanks, and these compounds were able to block *L. (L.) donovani* proliferation *in vitro*. Specifically, these compounds inhibit parasite dipeptidyl carboxypeptidase, which has been established as a putative target for antileishmanial chemotherapy [121].

By applying a similar strategy, the virtual screening of the ChemBridge databank for inhibitors of parasitic cysteine proteinases led to the identification of five non-peptide inhibitors with antileishmanial activity against *L. (L.) donovani* promastigotes *in vitro*. These inhibitors were selected by their potential capacity to bind to falcipain-2 and 3 from *Plasmodium* parasites, as measured through *in silico* assays, and their binding to *Leishmania* CPs can be explained by the high conservation of the CP binding pocket structure across these protozoans [122].

The small molecule thiocarbazole (PubChem SID 26681509) is another synthetic inhibitor that was discovered...
by analyzing the NIH Molecular Libraries Small Molecule Repository. It is a potent inhibitor of human cathepsin L and has demonstrated toxicity against *L. (L.) major* promastigotes, although it is safe to human aortic endothelial cells even at high concentrations [123].

A potent synthetic inhibitor of calpains, carbobezoxyz-valinyl-phenylalaninal (commercial name, MDL 28170), also presents antileishmanial activity. MDL 28170 induces *L. (L.) amazonensis* parasite death in culture and promotes alterations in the cell morphology. Possible targets for this inhibitor are the calpain-like molecules present on the cell surface of the promastigotes [124].

**Effects of Proteinase Inhibitors in the Treatment of Experimental Leishmania Infection**

Presently, little data are available in the literature regarding the outcome of using proteinase inhibitors targeting parasite enzymes to treat experimental *Leishmania* infections in experimental animal infections or in host cell cultures.

Nevertheless, CP inhibitors have been reported to have promising results when applied in a chemotherapeutic context in experimental animal infection models. Two derivatives of oxalic bis[(2-hydroxy-1-naphthyl) methylene]hydrazone (named ZLIII43A and ZLIII115A), both reversible CP inhibitors, and the pseudopeptide substrate analogue morpholine urea-phenylalanine-homophenylalanine-vinylsulfonyl-benzène (K11002, Arris Pharmaceuticals), an irreversible inhibitor of CP, were shown to interfere in *L. (L.) major* infections *in vitro* and *in vivo* [86]. These compounds prevented parasite replication and infection of mouse macrophages (cell line J774) challenged in culture with *L. (L.) major* promastigotes. However, none of these compounds affected the host cells (as assessed by analysis of morphological changes).

These same compounds delayed lesion progression and reduced the parasite burden in infected BALB/c mice, without toxic effects to the treated animals. Contrary to the mechanism of action previously suggested for another CP inhibitor (CAO74, a cathepsin B-specific inhibitor) that was also able to cure *L. (L.) major*-infected BALB/c mice [125], these inhibitors were not able to alter the T lymphocyte response of the host and appeared to act directly on the *Leishmania* CPs.

A distinct CP inhibitor, N-benzyloxy carbonyl-phe-aladiazomethylketone (Z-FA-DMK, Sigma), also impaired the parasite infection of host cells [126]. In this case, it has been suggested that Z-FA-DMK affects the activity of *L. (L.) mexicana* cysteine proteinase B, a known pivotal virulence factor for this species; however, the specificity of this action was not completely defined. This inhibitor was very efficient at preventing the infection of peritoneal cells from BALB/c mice by promastigotes or amastigotes and also reduced the number of infecting parasites per host cell; however, when used in parasite cultures for extended periods, it did not alter the growth of the parasite cells and only partially inhibited their CP activities.

The treatment of *L. (L.) tropica*-infected BALB/c mice with subcutaneous or intraperitoneal injections of an irreversible CP inhibitor, the pseudopeptide substrate analogue N-Pip-phenylalanine-homophenylalanine-vinyl sulphone phenyl (N-Pip-F-hF-VS Phenyl or K11777), showed that this compound possesses antiparasitic activity. The treated animals showed a reduction in lesion size, and this effect persisted for as long as 2 months after the end of treatment [127].

This same CP inhibitor, K11777, was also reported to hinder the survival of *L. (L.) mexicana* parasites inside peritoneal macrophages from CD1 mice [128]. When parasites were incubated with K11777 prior to the infection assays, macrophage infection rates fell by nearly 75%. This effect is comparable to what is observed in experimental challenges using mutant parasites with cpa and cpb gene depletion. The effect of this inhibitor appears to be related to preventing autophagosome digestion in the parasites.

Cystatin, another inhibitor of CPs, has also been reported to show activity in murine models of *Leishmania* infection, specifically in BALB/c mice infected with *L. (L.) donovani* [129]. The use of cystatin in conjunction with interferon (IFN)-γ demonstrated antiparasitic effects. This combination affected amastigote growth inside macrophages *in vitro* and had curative effects for the infected animals, including the virtual elimination of parasites in the spleen after treatment. Interestingly, such treatment also led the treated mice to develop subsequent resistance to infection challenge. This evidence suggests the possibility that this CP inhibitor is not acting directly on parasite enzymes but is rather affecting the host cells in some way. This hypothesis is supported by the observations that macrophage nitrite production is enhanced, and the T lymphocyte response is altered by cystatin.

Two compounds derived from *trans-aziridine-2,3-dicarboxylic* acids (13b and 13e), which are irreversible inhibitors of cathepsin B-like enzymes, have been reported to reduce the *L. (L.) major* infection rate in peritoneal macrophages obtained from BALB/c mice without showing toxic effects against the mammalian cells. These compounds induce parasite death with features that are similar to apoptosis, namely the accumulation of undigested debris in lysosome-like vacuoles within the parasites [130]. Similar to the pattern observed for cystatin, co-treatment with IFN-γ enhanced the antiparasitic effects of these compounds in infected macrophages. Furthermore, compounds 13b and 13e interfered with the production of interleukins and NO in infected peritoneal macrophages. These compounds changed the levels of IL-6, IL-12 and tumor necrosis factor (TNF)-α in cell culture and increased NO levels [131].

CP inhibitors have also demonstrated potential in the treatment against parasites during *Leishmania* infection of human cells or tissues. The cathepsin B-specific inhibitor CAO74, which had a protective effect for BALB/c mice against *L. (L.) major* as described above, was shown to interfere in the infection of macrophages from the human myeloid cell line U937 with *L. (L.) infantum*. CAO74 reduced parasite survival inside host cells [132]. Remarkably, the mechanism of this effect appears distinct from the one observed in the murine host and is possibly related to the inhibition of the parasite CPs rather than a direct effect on the host enzymes. In addition, because *Leishmania* cathepsin B has been implicated in the activation of host transforming growth factor (TGF)-β, which leads to an inefficient Th2...
response, the inhibition of this enzyme by CA074 would prevent TGF-β activation and allow for the host to control the infection through an effective Th1 response.

Specific inhibitors of another class of CPs, cathepsin L, have also been shown to abrogate the parasite effect on IL-12 expression [133]. The compound cathepsin L inhibitor IV (Calbiochem) has been reported to interfere in the parasite-related cleavage of nuclear factor (NF)-κB or its endogenous inhibitors, as assayed in bone marrow-derived macrophages challenged with L. (L.) mexicana. This effect prevents IL-12 production by macrophages even after lipopolysaccharide (LPS) stimulation. Similar results have also been observed for K11002, which was previously mentioned due its effects on L. (L.) major survival in infected mice or murine cell cultures.

Inhibitors of other proteinase classes also have the potential to be used for the treatment of Leishmania infection. Five aspartyl proteinase inhibitors that are currently used for the treatment of HIV infection (amprenavir, indinavir, lopinavir, nelfinavir and saquinavir) have been shown to affect L. (L.) amazonensis parasites in vitro, although with varying degrees of activity. These inhibitors impaired parasite growth in culture and induced changes in cell morphology. In addition, amprenavir, lopinavir and nelfinavir interfered with the parasite-macrophage association indexes if they were incubated with the promastigotes prior to the interaction assays [116].

However, other studies have reported that indinavir and ritonavir could not effectively control the infection in L. (L.) amazonensis experimentally infected mice; these antiretrovirals were able to reduce lesions in infected mice after 3-5 weeks of treatment, but they did not affect the parasite load [134].

Serine proteinase inhibitors present in potato tuber extract, which were effective to inhibit SP activities in log-phase promastigotes of L. (L.) donovani, also reduced viability of promastigotes in culture and affected amastigotes proliferation inside murine peritoneal macrophages [135]. This extract presented the potential to serve as basis for the development of new drugs against visceral leishmaniasis, as it was shown to have no adverse effects on macrophages, while enhancing the production of nitric oxide and reactive oxygen species, molecules related to parasite killing, in these cells.

REMARKS

Leishmaniasis is a neglected tropical disease and, as this term implies, there is a great need for additional research to improve the treatment options for this disease. Antimonials are the current first-choice drugs for leishmaniasis treatment and have been so for a substantial period of time. However, they are highly toxic compounds and, if they were proposed currently as a new drug, they would likely not be approved due to the rigid eligibility criteria and strict regulatory aspects for clinical trial investigations for drug approval [136, 137].

The current trends for drugs research in parasitic diseases include the application of proteinase inhibitors as chemotherapeutic agents. However, the data collected in independent reports have been unable to effectively prove that proteinase inhibitors can adequately replace antimonials in the treatment of leishmaniasis.

As previously described for L. (L.) amazonensis [81], Leishmania parasites differentially express proteinases in the various stages of their life cycle, reflecting the changes in their environment. These variations must be regarded when considering proteinase inhibitors as potential chemotherapeutic drugs. New drugs should focus primarily on the proteinases that are relevant to the parasite morphological stages that inhabit vertebrate hosts.

Another important point to consider is that certain minor structural differences in proteinases of the same type can affect their catalytic site microenvironment. These variations may make it difficult to develop proteinase inhibitors with wide activity against an array of proteinases in Leishmania spp.

In addition, due to the large variety of proteinases reported in these parasites, it is possible that only a combined treatment with distinct proteinase inhibitors would be an effective antileishmanial therapy.

Finally, it is important to consider that, despite the relative safety that proteinase inhibitors have been presenting when used against Leishmania in cell cultures or animal models, these drugs may cause some undesired side effects when applied in the treatment of leishmaniasis patients. It has been reported that proteinase inhibitors used in anti-HIV therapies may cause dyslipidemia, insulin resistance, type 2 diabetes, cardiac conduct abnormalities, nausea and diarrhea, however the more recently developed inhibitors cause these side effects less frequently [138-141]. Currently, an experimental animal model, using hamsters, is being developed to conduct studies about the side effects of antiretroviral proteinase inhibitors [141] and may, therefore, be also useful to determine the safety of potential antileishmanial proteinase inhibitors.

We firmly believe that only by understanding the modulation of distinct isoforms of the same proteinase will it be possible to propose a fine-tuned and effective strategy for controlling Leishmania parasites during infection of the vertebrate host. The establishment of treatments based on such variations would be a great contribution to controlling and curing this relevant parasitic disease.

<table>
<thead>
<tr>
<th>Proteinases are a promising target for developing drugs to treat leishmaniasis because:</th>
<th>Relevant aspects to be considered for the development of treatments based on Leishmania proteinase inhibitors:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• They are pivotal factors for parasites’ life cycle and interaction with host;</td>
<td>• Parasites express distinct sets of proteinases in different phases of their life cycle;</td>
</tr>
<tr>
<td>• Drugs currently available for leishmaniasis present many toxic side effects and resistant strains are emerging;</td>
<td>• Minor structural differences in the catalytic site of proteinases of a same type may prevent broad effect by the inhibitors;</td>
</tr>
</tbody>
</table>
CONFLICT OF INTEREST

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

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