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Review article

Leishmanicidal therapy targeted to parasite proteases

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ABSTRACT

Leishmaniasis is considered a serious public health problem and the current available therapy has several disadvantages, which makes the search for new therapeutic targets and alternative treatments extremely necessary. In this context, this review focuses on the importance of parasite proteases as target drugs against *Leishmania* parasites, as a chemotherapy approach. Initially, we discuss about the current scenario for the treatment of leishmaniasis, highlighting the main drugs used and the problems related to their use. Subsequently, we describe the inhibitors of major proteases of *Leishmania* already discovered, such as Compound s9 (aziridine-2,3-dicarboxylate), Compound 1c (benzophenone derivative), Au2Phen (gold complex), AubipyC (gold complex), MDL 28170 (dipeptidyl aldehyde), K11777, Hirudin, diazo-acetyl norleucine methyl ester, Nelfinavir, Saquinavir, Nelfinavir, Saquinavir, Indinavir, Saquinavir, GNF5343 (azabenzoxazole), GNF6702 (azabenzoxazole), Benzamidine and TPCK. Next, we discuss the importance of the protease gene to parasite survival and the aspects of the validation of proteases as target drugs, with emphasis on gene disruption. Then, we describe novel important strategies that can be used to support the research of new antiparasitic drugs, such as molecular modeling and nanotechnology, whose main targets are parasitic proteases. And finally, we discuss possible perspectives to improve drug development. Based on all findings, proteases could be considered potential targets against leishmaniasis.

1. Introduction

Leishmaniasis is a disease caused by protozoa of the genus *Leishmania* and is transmitted to humans and other mammals through the bite of insect vectors [1]. The life cycle of *Leishmania* parasites is comprised by two evolutionary stages: (1) intracellular amastigotes inside macrophages of the mammalian hosts and (2) extracellular promastigotes in the gut of the insect vector [2]. About 53 *Leishmania* species have been described, of which 31 can infect mammals and 20 are considered pathogenic to humans [3]. The common clinical signs may be grouped in: cutaneous leishmaniasis (CL), cutaneous diffuse leishmaniasis (DCL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (VL) [3,4]. CL is the most common form of the disease, while VL is its most serious form [5]. It is believed that more than one billion people live in endemic areas at risk of infection, of which, about 616 million and 431 million for VL and CL, respectively. A total of 300,000 cases of VL are estimated annually, with > 20,000 deaths per year. Regarding CL, one million of cases were estimated in the last five years [6]. These data make leishmaniasis a serious public health problem [3].

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There are no effective vaccines against leishmaniasis in humans at present, and consequently, the disease control is primarily based on chemotherapy [7]. However, none of the available drugs can be considered ideal due to toxicity, long-term treatment, severe adverse effects [8], high cost, invasive administration routes and low effectiveness [9]. Therefore, there is an urgent need for novel compounds with significant leishmanicidal effect and low toxicity to the host.

Proteases are enzymes adapted throughout evolution that hydrolyze protein substrates [10]. According to their catalytic activity, proteases can be classified as serine proteases (S), cysteine proteases (C), aspartic proteases (A), metalloproteases (M), threonine proteases (T), glutamic proteases (G), asparagine lyase proteases (N), mixed catalytic-type peptidases (P), protein inhibitors (I) and peptidases of unknown catalytic type (U) [11]. Several studies indicate that *Leishmania* proteases are involved in tissue host invasion, survival inside macrophages and host immune response modulation, which make them important virulence factors against these parasites (discussed during the review). This fact draws attention to *Leishmania* proteases as potential anti-parasitic therapeutic targets.

Pathways for the development of new drugs based on parasite proteases require the target protein validation, in order to identify the importance of the corresponding gene to the parasite survival. Thus, the protein structure should be elucidated by experimental methods or predicted by computational tools, followed by screening of selected suitable compounds that can be tested for their biological activity. If the compound has the desired effects, it can be evaluated in a murine model of leishmaniasis. If the compound does not have a satisfactory effect or if it is toxic for the host cells, this molecule can be optimized by medicinal chemistry strategies or by vectorization, for example, using nanoformulations (Fig. 1).

In this work, we outline important aspects of the current leishmaniasis chemotherapy. In order to overcome the problems related to available medicines, we will show some parasite protease inhibitors described in the literature and strategies to develop new treatments.

2. Current leishmaniasis chemotherapy

First-line chemotherapy to treat most cases of leishmaniasis is based on parenteral administration of pentavalent antimony (Fig. 2) [12,13]. The medicinal use of antimony compounds has been known since their introduction by the alchemist John of Rupescissa in the 14th century; however, only in 1912, Gaspar de Oliveira Vianna observed the effectiveness of trivalent antimony (Sb^{+3}) or emetic tartar in CL therapy. Three years later, in Italy, its use was also demonstrated in the treatment of VL [14]. However, this medicinal product causes several toxic and undesirable effects, and for this reason, it was replaced by pentavalent antimony (Sb^{+5}) compounds. In 1920, Bramachari developed the first Sb^{+5} compound, urea stibamine, which showed less toxicity than Sb^{+3} [14] and emerged as an effective therapy against VL in India [15]. Currently, pentavalent antimony is available in two different formulations, Glucantime® and Pentostam®, but only the first is available in Brazil [16]. These drugs have an effectiveness of about 90% [17]. However, variable efficacy may occur, depending on the species of *Leishmania* [18], geographic region [16], presence of resistant strains and therapeutic regimens. In Brazil, 16% of the patients did not respond to treatment [19]. Additionally, the lengthy period of treatment and the high incidence of adverse effects (Table 1) limited the use of these drugs in the clinical practice [12].

Despite the long-established use of pentavalent antimony in leishmaniasis treatment, the mechanism of action of these drugs in *Leishmania* has not been fully elucidated. For example, it is not known if the active form is Sb^{+5} or $Sb^{+3.}$ Therefore, three models of mechanism of action are proposed for pentavalent antimonials: (1) the compounds act as pro-drugs, (2) they have intrinsic leishmanicidal activity and (3) they induce host immune system activation [15] (Table 1).

In cases that are refractory to pentavalent antimony, or in which it cannot be used, second-choice drugs can be employed, such as amphotericin B, pentamidine and miltefosine [20]. Amphotericin B (Fig. 2) is an antifungal agent [21] that can be parenterally used for treating leishmaniasis [21,22]. It is effective against different species of *Leishmania* and is the first-choice treatment for pregnant and patients co-infected with human immunodeficiency virus (HIV)/*Leishmania* [23]. The efficacy of this drug is above 90% [14,24]. However, as pentavalent antimony, amphotericin B presents high toxicity (Table 1), requiring hospitalization [7]. These facts prevent its use as the first-choice drug for the treatment of leishmaniasis [25]. Table 1 discloses the mechanism of action of amphotericin B.

In order to reduce the toxicity of amphotericin B, lipid formulations have been developed, like AmBisome®, Amphocil® and Abelcet® [12]. These formulations can be used to treat leishmaniasis and are less toxic when compared with non-liposomal amphotericin B [26]. A phase III Clinical Trial is being conducted in Bahia, Brazil, to assess the efficacy of liposomal amphotericin in patients with disseminated leishmaniasis, a severe and emerging form of CL in the Americas that presents high failure rates when treated with the first-line therapies. Liposomal amphotericin demonstrated a cure rate of 75% at doses > 30 mg/Kg, which is considered effective for treating disseminated leishmaniasis [27]. Other Clinical Trial was also performed in Brazil to define what could be the best treatment regimen for VL in the country, since there is an urgent need to improve the current treatment. This study evaluated the efficacy and safety of amphotericin B deoxycholate (for 14 days); liposomal amphotericin (for 7 days) and one combination of liposomal amphotericin and meglumine antimoniate in a single dose for 10 days, compared with the standard treatment with meglumine antimoniate for 20 days. The treatment with amphotericin B deoxycholate was discarded due to its high toxicity observed during this regimen. Regarding the cure rates obtained with the other groups, there was no statistically significant difference between the experimental procedures (cure rates: liposomal amphotericin alone = 87.2%; combination of liposomal amphotericin and meglumine antimoniate = 83.9% and meglumine antimoniate alone = 77.5%). However, the treatment with liposomal amphotericin caused less adverse effects when compared with standard treatment, and therefore, the use of liposomal amphotericin was considered by the authors as a more suitable first-line treatment against VL in Brazil [28]. In a recent paper, the effect of three distinct delivery systems of amphotericin B (polymeric micelle system, Ambisome® and amphotericin B deoxycholate) was evaluated in a murine model of VL. The polymeric micelle system and Ambisome® presented the best results, with significant reduction of parasite load, helping the development of a parasite-specific Th1 immune response and without hepatic or renal damage. The treatment with amphotericin B deoxycholate and Glucantime® caused significant toxicity to infected animals [29]. Nevertheless, although studies indicate the use of liposomal amphotericin in the treatment of leishmaniasis, this drug is expensive, thus limiting its use [12].

Pentamidine (Fig. 2) is another leishmanicidal drug administered by intravenous or intramuscular route [30,31] that is effective against leishmaniasis in the treatment of patients that do not respond to pentavalent antimony [32,33]. However, its efficacy varies according to geographic location and the species involved [31]. Additionally, the use of pentamidine is also inadequate as a first-line treatment due to its high toxicity (Table 1) [12]. Table 1 discloses the action mechanism of pentamidine.

Miltefosine (Fig. 2) was developed as an antineoplastic agent to treat cutaneous tumors and has been used to treat leishmaniasis [34]. So far, this drug is the only one administered by oral route for treating these diseases [35]. In a study conducted in India involving > 1100 patients with VL, this drug had an efficacy index of 95% [36,37]. The oral administration of miltefosine has been used for treating VL in India since 1998 and it is also recommended in Ethiopia, Colombia, Bolivia and Guatemala to treat CL [38]. However, miltefosine efficacy seems to vary depending on the species of *Leishmania* [39]. In murine model, for

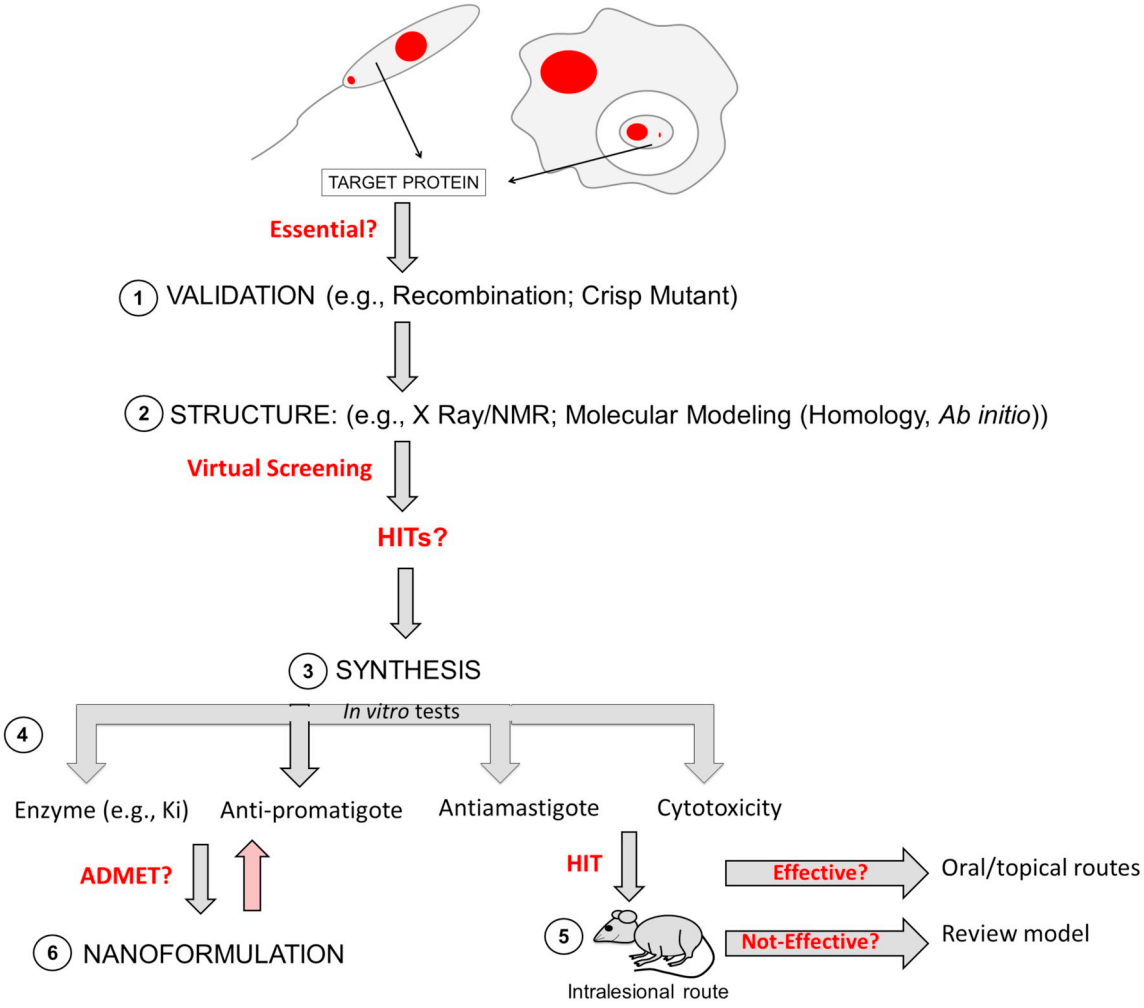


Fig. 1. Proposed pathway for the discovery of new drugs based on *Leishmania* proteases as potential targets. After identifying a target protein, the following step is to validate if this protein is, in fact, an essential gene to the parasite (1); then, the protein structure must be elucidated or built by computational modeling. The virtual screening can be performed using compound databases available (2); the next step is the synthesis of promising compounds, for example, to interfere or alter the protein structure or functionality (3); the compounds should be evaluated for their anti-parasite effectiveness and lack of cytotoxicity to the host to identify hits. Examples of protocols are the effective evaluation of recombinant enzymes and anti-*Leishmania* promastigote and amastigote assays (*in vitro* effect) (4); if a compound presents a good potential antileishmanial activity and low toxicity to macrophages, it should be tested *in vivo* (murine model of leishmaniasis) (5); if there is any inadequate ADMET parameter, such as absorption or host toxicity, nanoformulations can be prepared (6); the nanoformulation should follow steps 4 and 5.

example, disease recurrence was observed after treatment of BALB/c mice infected with *L. amazonensis* [40] and *L. braziliensis* [39]. Moreover, it has been reported that, for *L. amazonensis*, *L. braziliensis*, *L. guyanensis* and *L. chagasi*, species prevalent in Brazil, miltefosine was not effective, and for this reason, high doses of this drug would be necessary to treat patients infected with these species [41]. Refer to Table 1 for action mechanism and adverse effects of miltefosine.

New treatment alternatives for leishmaniasis are being studied to reduce toxicity and replace the administration routes of the treatments currently available. In this context, a phase III Clinical Trial showed cure rates of 77.8% and 78.6% for topical cream containing 15% paromomycin and topical cream containing 15% paromomycin plus 0,5% gentamicin, respectively. Both were used once daily for 20 days in patients with CL caused by *L. panamensis*. This administration route reduces the possibility of systemic adverse effects and is more comfortable to the patient, thus reducing the rate of treatment withdrawal [42]. The combined therapy is another alternative that is under study. Recently, the combined effects of Glucantime® plus amiodarone and Glucantime® plus itraconazole for treating hamsters infected with *L. amazonensis* were shown. Amiodarone and itraconazole improved Glucantime® activity during the treatment, which was evidenced by a reduction of the size of lesions when compared with the animals treated only with Glucantime®. This allows reducing the dose of the first-line treatment in order to minimize its side effects [43]. These studies are very satisfactory, but until now, there are not novel therapeutic strategies that can replace the first and second-line chemotherapy to treat leishmaniasis.

Hence, in face of the difficulties related to the current treatment of leishmaniasis, the discovery of new therapeutic agents is mandatory. In this context, proteases emerge as potential new drug targets in *Leishmania* parasites.

3. Proteases as a drug target

Proteases are involved in both physiological and pathological processes. They are considered a good target to drug development, since their structure and enzymatic mechanisms are well known [56]. One of the most known protease-based therapies uses the HIV protease inhibitors, which are widely used in the combined treatment of AIDS. Currently, there are 10 HIV protease inhibitors approved by FDA, including saquinavir, ritonavir, indinavir, nelfinavir, lopinavir, atazanavir, amprenavir, fosamprenavir, tipranavir and darunavir (Fig. 3) [57,58].

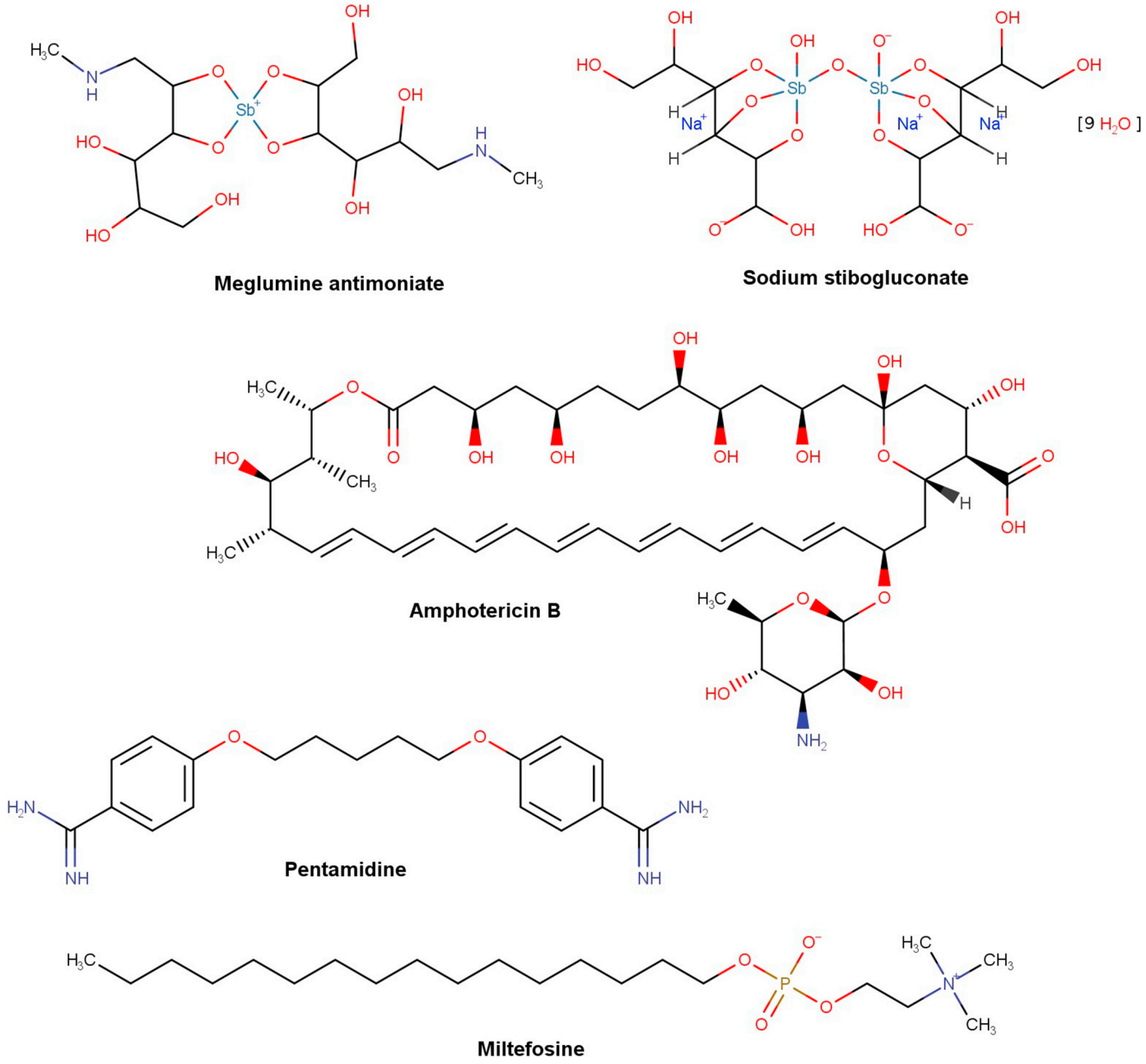


Fig. 2. Chemical structures of drugs currently used in leishmaniasis chemotherapy.

Since HIV proteases inhibitors have peptide moieties, they cause ADME-related problems, like poor oral bioavailability. To overcome these issues, prodrugs [59–61] and novel nanoformulations [62,63] were developed, improving resistance to oral administration and reducing adverse effects [61,64].

In parasitic diseases, the proteases can be also considered potential drug targets. Maretti-Mira and colleagues demonstrated that macrophages from individuals cured of ML have increased matrix metalloproteinase-9 (MMP-9) activity compared with macrophages from individuals cured of CL, thus correlating MMP-9 with the occurrence of ML [65]. In VL, high levels of MMP-9 in serum were detected and, although they were initially associated with an inflammation increase, it was observed that this biomarker can be used to monitor the success of therapy [66]. MMP-9 is an important factor to regulate excessive inflammation during VL [67]. In CL caused by *L. braziliensis*, high levels of MMP-2 were associated with a satisfactory response to pentavalent antimonial treatment [68].

3.1. Proteases as target drugs against leishmaniasis

Proteases are considered virulence factors involved in the pathogenesis of protozoa, such as *Leishmania* and *Trypanosoma* [69]. Proteases from parasites facilitate host cells invasion, metabolism of host proteins and evasion of the host immune system [70]. In *Leishmania*, the protease importance has been confirmed by the discovery of specific protease inhibitors, which are able to eliminate parasites and reduce the evolution of lesions caused by them [71]. Among the *Leishmania* species, there is a difference in relation to the content of protease genes. In *L. braziliensis*, for example, protease genes represent 2.18% of total genes. This value varies to 1.61% in *L. infantum*, 1.52% in *L. mexicana* and 1.41% in *L. major*. In *L. braziliensis*, the metalloprotease genes accounts for 52% of total protease genes and this varies to other *Leishmania* species, such as 40% in *L. infantum* and 35% in *L. major* and *L. mexicana*. Among these species, the percentage of cysteine and serine protease genes is similar, representing 36 to 47% and 10 to 16% of total

Table 1
Drugs currently used in the treatment of leishmaniasis, its adverse effects and action mechanism.

Drug	Adverse effects	Action mechanism
Pentavalent antimony	Cardiotoxicity [44], arthralgia, anorexia, fever, urticaria and significant toxicity to liver, kidneys and spleen [12]. Hospitalization and constant monitoring of patients during treatment are needed [24].	(1) First model: Sb^{+5} compounds act as a pro-drug (Sb^{+5} is reduced to a most potent form against the parasite, $Sb^{+3)}$.[15]. This reduction seems to occur within macrophages [20]. It explains why amastigotes are susceptible to the action of Sb^{+5}, while promastigotes have low susceptibility [15]. Sb^{+3} inhibits the trypanothione reductase (TR) enzyme, which leads to oxidative stress inside the parasites [20,45]. (2) Second model: Sb^{+5} is active against *Leishmania* which acts through the inhibition of glycolysis and beta-oxidation of fatty acids. Sb^{+5} also seems to interfere in the DNA replication of the parasites [15]. (3) Third model: host immune system is activated by Sb^{+5} (mechanisms of innate and adaptive immunity) [15]. Innate immunity: it has been demonstrated that pentavalent antimonials can induce the production of nitric oxide (NO) and reactive oxygen species (ROS) by murine macrophages infected by *L. donovani* amastigotes [46]. Adaptive immunity: murine model studies of VL have shown that pentavalent antimony requires a population of Th1 cells to cause a leishmanicidal effect [47].
Amphotericin B	Nephrotoxicity, cardiac alterations, hemolysis, liver damage, nausea and fever [12].	Binding to ergosterol present in the parasite plasma membrane, which causes structural disorganization and create pores on the cell membrane, resulting in ionic imbalance and death [20,22,48]. It can also act by auto-oxidation, leading to the production of free radicals [49]. Although it has not been described for *Leishmania*, the amphotericin B "sponge model" in yeast proposes that this compound extracts ergosterol from bilayer lipid membranes by producing large aggregates, killing the microorganism [50]. It can also recognize, to a lesser extent, cholesterol in human cells, thus explaining its side effects [51].
Pentamidine	Hyperglycemia (as a result of pancreatic damage), hypotension, tachycardia [24], nephrotoxicity and hepatotoxicity [4].	Affects the parasite DNA synthesis, modifying the kinetoplast morphology and promoting mitochondrial membrane fragmentation, leading to parasite death [16]. Yang and colleagues described the selectivity of pentamidine to kinetoplast DNA and not to nuclear DNA in *L. donovani* [52].
Miltefosine	Teratogenicity, and therefore, it should not be prescribed to pregnant or breastfeeding women [12].	Causes disorders in the lipid metabolism of *Leishmania*; inhibits the cytochrome C-oxidase enzyme, causing mitochondrial damage; exerts an immunomodulatory effect on cells infected with this protozoan [53] and induces cell death by apoptosis in *Leishmania* [54]. Recently, it was shown that it induces unbalance of the lipid content of the plasma membrane of *Leishmania*, causing decrease of phospholipids and increase of sphingolipids and sterols [55].

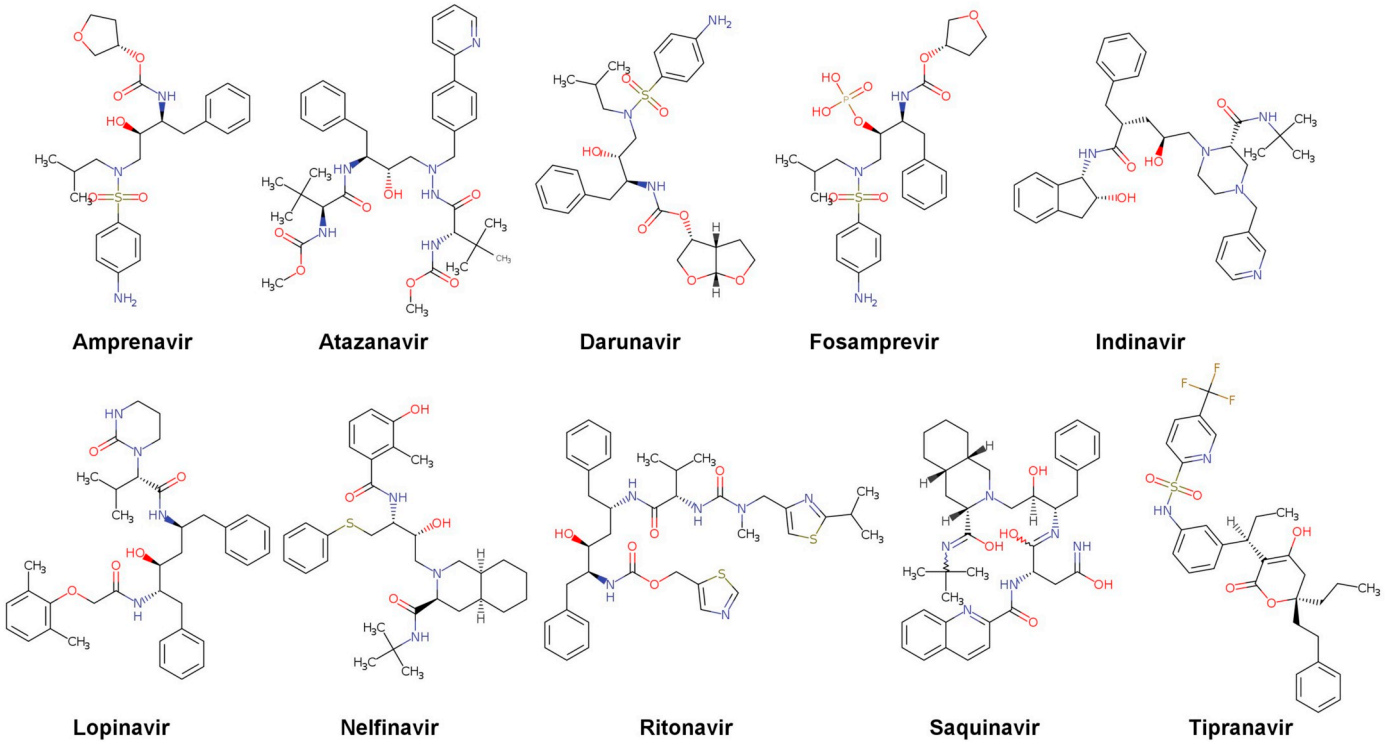


Fig. 3. Chemical structures of HIV protease inhibitors approved by FDA.

protease genes, respectively. Regarding aspartic protease genes, it is believed that they account for only 3% of protease genes [72].

A review published in 2013 discusses the potential use of protease inhibitors in the development of leishmaniasis drugs focused in cells biology and the potential use of peptidases inhibitors as chemotherapy. In this document, the authors cite drug targets in Leishmania, highlighting the enzymes as the most important targets, due to their regulatory role in metabolic and biochemical pathways. According to this paper, many enzymes can be considered target drugs in this protozoan, such as dihydrofolate reductase, trypanotione system, protein kinases, topoisomerases, metacaspases, including proteases. Posteriorly, the authors discuss some classes of important proteases in *Leishmania* and cite some inhibitors already described in the literature [73]. They showed the effect of synthetic and natural (*Leishmania* Cysteine Protease Inhibitor – ICP; Ecotilin-like serine peptidase inhibitors – ISP) peptidase inhibitors at promastigotes showing the effect on growing, ultrastructural biology and infectivity. Only few cases about treatment of infected macrophages and *in vivo* treatment were addressed.

In a different approach, the present review is a robust searching of a high numbers of molecules, and therefore, we updated the novel molecules, protease inhibitors, found in the literature and used resources of molecular biology, molecular modeling and nanotechnology as power tools to find novel drugs, which are addressed below. This review focuses on serine proteases, cysteine proteases, metalloproteases, aspartic proteases and proteasomes, as will be discussed below. For each class of protease, different inhibitors have been already described and some of them are summarized in Table 2 and in Fig. 4.

3.1.1. Serine proteases

Within the proteases, serine proteases constitute one of the major enzymatic groups [74]. These enzymes are involved in the pathogenesis of a number of infectious diseases, including parasitic diseases [70], in which these enzymes play an important role in the parasite-host interaction, and consequently, they may represent important targets to the chemotherapy of these diseases [75,76].

In *Leishmania*, the highest proteolytic activity identified is attributed to the serine protease oligopeptidase B (OPB) [69]. Two OPBs (OPB and OPB2 - also called OPB-like) were identified in *Leishmania major* [77–79] and in *L. amazonensis* [79,80]. In *Trypanosoma cruzi*, OPB is mainly involved in parasite invasion of the host cell [81,82]. In *Leishmania*, *L. major*-OPB knockout parasites showed reduced capacity to infect and proliferate in host macrophages [82,83]. OPBs can be considered ideal targets in *Leishmania,* since they are expressed in both evolutionary forms of this parasite (promastigotes and amastigotes) and, additionally, no homologues of these enzymes have been identified in humans [79,80]. In African trypanosomiasis, caused by *Trypanosoma brucei*, the first choice chemotherapy is based on pentamidine, which causes reduction of oligopeptidase activity [76]. Interestingly, pentamidine may also be employed in the treatment of leishmaniasis as a second choice drug [23,30] which suggests that OPBs are important targets in *Leishmania* [80].

Other serine protease already identified in *Leishmania* is subtilisin, and this enzyme has been also shown to be an important therapeutic target in this protozoan [84]. *L. donovani* subtilisin was studied by Swenerton and colleagues. They developed a transgenic parasite which showed that the lack of this protease impairs the differentiation of promastigotes in amastigotes, reduces the virulence of the parasite and increases its sensitivity to oxidative damage [84]. These factors highlight the importance of subtilisin during the biological cycle of *Leishmania*. In *L. major*, only one allele in the subtilisin gene could be silenced, showing the extreme importance of this protease in the parasite survival [84]. Thus, subtilisin can be also considered a therapeutic target in this protozoan.

Signal peptidase (SPase) is another serine protease that has an important role in *Leishmania* parasites, since it was not possible to create a *L. major* null mutant. Moreover, the heterozygote mutant showed a reduced level of infectivity and was unable to cause lesion in BALB/c mice [85]. This fact suggests that this serine protease is an important therapeutic target in *Leishmania* protozoan.

The importance of serine proteases to these parasites and the possibility of using them as therapeutic targets in parasitic diseases drew the attention of several researchers and motivated them to find new protease inhibitors [86–88]. Silva-Lopez and colleagues evaluated the leishmanicidal effect of some serine protease inhibitors. Two classical inhibitors (Fig. 4), benzamidine (Bza) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which are important inhibitors of *L. amazonensis* serine protease, were effective in reducing the promastigotes viability. Additionally, these inhibitors induced changes in the flagellar pocket and membrane, such as bleb formation. Furthermore, a substance obtained from sea anemone *Stichodactyla helianthus* (ShPI-I) has proven to be a good inhibitor of *L. amazonensis* serine proteases, impairing the parasites viability. ShPI-I (Fig. 4) causes more changes in the ultrastructure of *L. amazonensis* promastigotes than the classical inhibitors. These changes include vesicle formation on the flagellar pocket membrane and in the cytoplasm (similar to autophagic vacuoles) and alterations of the promastigote shape [89]. Other work, published by Souza-Silva and colleagues, showed the effect of epoxy-α-lapachone (Fig. 4), a naphthoquinone derivative, on *L. amazonensis*. This compound caused significant inhibition of *L. amazonensis* serine proteases. Furthermore, epoxy-α-lapachone was effective against promastigote and amastigote forms of this *Leishmania* species and affected plasma membrane organization, according to data shown by Silva-Lopez and colleagues. This protease inhibitor also showed *in vivo* effect, causing reduction of the lesion size in mice infected with *L. amazonensis*. Interestingly, *in silico* studies showed that epoxy-α-lapachone can bind to OPB of *L. amazonensis* similarly to antipain, a protease inhibitor [90]. Das and colleagues studied *Coccinia grandis* leaf extract and identified as serine protease inhibitors which significantly inhibited *L. donovani* serine proteases in enzymatic assays. These inhibitors showed significant antileishmanial effects with IC_{50} (50% inhibitory concentration) at 308 μg/mL on *L. donovani* promastigotes (Table 2) [91]. Paik and colleagues obtained fractions rich in serine protease inhibitors from potato tuber, which showed strong inhibitory activity on serine proteases of *L. donovani* that were effective against promastigotes (IC_{50} = 312.5 μg/mL) and amastigotes (IC_{50} = 82.3 μg/mL) and induced ROS and NO production in macrophages infected by *L. donovani*. Interestingly, the potato tuber extract had no significant toxicity on macrophages (Table 2) [92].

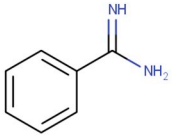
3.1.2. Cysteine proteases

Cysteine proteases have an important role in the life cycle of parasites, such as helminths (*Schistosoma*) and protozoa (*Plasmodium*, *Trypanosoma brucei*, *T. cruzi*, *Leishmania* and *Giardia duodenalis*) [93,94], playing different functions, such as protein processing, nutrition [93,95], host invasion [93,96], evasion from the host immune system [93] and virulence factor [94]. The most important cysteine protease from *T. cruzi* is cruzain (also known as cruzipain) [93,97] that has an essential role in all life cycle stages of this protozoa [98]. Inhibitors of this enzyme have shown curative effect in *in vitro* and *in vivo* models of Chagas disease [93,99]. In *L. tropica*, the use of a specific cysteine protease inhibitor, N-Pip-F-hF-*VS* Phenyl (K11777) (Fig. 4), showed that cysteine proteases are essential for the growth and pathogenicity of these parasites [100]. Kanaji and colleagues showed that squamous cell carcinoma antigen 1 (SCCA1) significantly inhibited the cysteine proteases from *L. mexicana* (CPB2.8), *T. cruzi* (cruzain) and *T. brucei rhodesiense* (rhodesain) [101]. In a screening from a series of aziridine- 2,3-dicarboxylate compounds, known as inhibitors of the cysteine protease cathepsin L, Schad and colleagues discovered some compounds with significant effect on promastigotes and amastigotes of *L. major*. Aziridine-2,3-dicarboxylate compound, a *N*-acylated *trans*-aziridine-2,3-dicarboxylate, named compound s9 (Fig. 4), was the best, showing selective inhibition of cysteine proteases of *L. major* (cathepsin

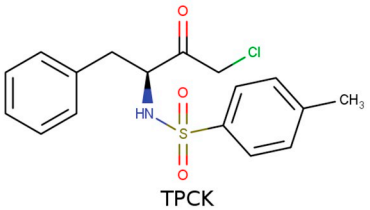
Table 2
Review of some protease inhibitors already described in the literature.

Inhibitor	Target	Concentration *in vitro* (IC_{50}[a])	Species	Adverse effects	Reference
Coccinia grandis leaf extract	Serine protease	Promastigotes - 308 μg/mL	*L. donovani*	N.D.[c]	[91]
Potato tuber extract	Serine protease	Promastigotes - 312.5 μg/mL Amastigotes - 82.3 μg/mL	*L. donovani*	No adverse effect to murine macrophages until 2,5 mg/mL	[92]
Compound s9 (aziridine-2,3-dicarboxylate)	Cathepsin B (cysteine protease)	Promastigotes - 37.4 μM Amastigotes - 2.3 μM	*L. major*	Not affected mammalian cathepsin L	[102]
Compound 1c (benzophenone derivative)	Cysteine proteases	Amastigotes - 74,4 μM	*L. amazonensis*	Murine macrophages - CC_{50}[d] = 496,1 μM	[103]
Au_2Phen (gold complex)	Cysteine protease	Amastigotes - 3.17 μM	*L. infantum*	Human fibroblast - IC_{50} = 2.16 μM	[104]
AubipyC (gold complex)	Cysteine protease	Amastigotes - < 0.25 μM	*L. infantum*	Human fibroblast - IC_{50} = 0.42 μM	[104]
MDL 28170 (dipeptidyl aldehyde)	Calpain (cysteine protease)	Promastigotes - 23,3 μM	*L. amazonensis*	N.D.[c]	[111]
Hirudin	GP63 (metalloprotease)	Promastigotes - 0.6 μg/mL Amastigotes - 0.43 μg/mL	*L. tropica*	Human macrophages - CC_{50}[d] = 860.11 μg/mL	[123]
DAN[b]	Aspartic protease	Promastigotes - 22 μM	*L. mexicana*	N.D.[c]	[126]
Nelfinavir	Aspartic protease	Promastigotes - 14.05 μM	*L. infantum*	N.D.[c]	[129]
Saquinavir	Aspartic protease	Promastigotes - 55.12 μM	*L. infantum*	N.D.[c]	[129]
Nelfinavir	Aspartic protease	Promastigotes - 9.85 μM	*L. mexicana*	N.D.[c]	[129]
Saquinavir	Aspartic protease	Promastigotes - 42.08 μM	*L. mexicana*	N.D.[c]	[129]
Indinavir	Proteasome	Promastigotes - 8.3 μM	*L. major*	N.D.[c]	[139]
Saquinavir	Proteasome	Promastigotes - 7.0 μM	*L. major*	N.D.[c]	[139]
GNF5343 (azabenzoxazole)	Proteasome	Amastigotes - 7.3 μM	*L. donovani*	N.D.[c]	[140]
GNF6702 (azabenzoxazole)	Proteasome	Amastigotes - 18 nM	*L. donovani*	No inhibition of mammalian proteasome or the growth of mammalian cells, and is well-tolerated in mice	[140]

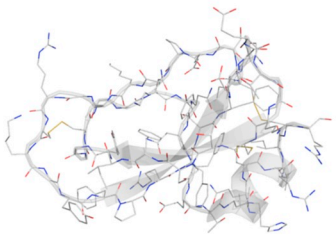
[a] IC_{50} = inhibitory concentration of 50% parasitic growth.
[b] DAN = diazo-acetylnorleucinemethylester.
[c] N.D. = not determined.
[d] CC_{50} = cytotoxic concentration of 50% of cells.



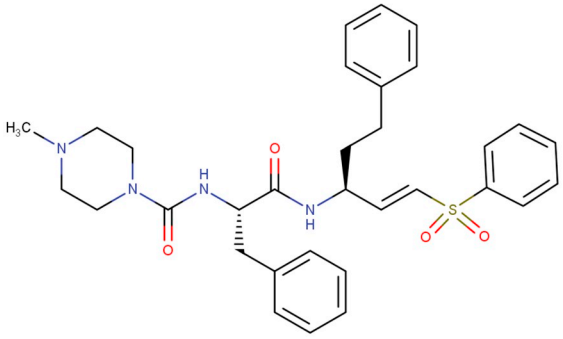
BZA
(Benzamidine)



TPCK
(N-*p*-Tosyl-L-phenylalanine chloromethyl ketone)



ShPI-I
(PDB code 3T62, Garcia-Fernandez)



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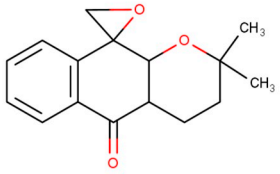
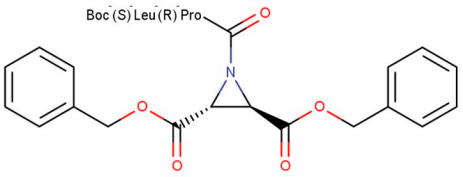
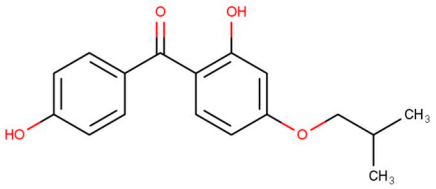


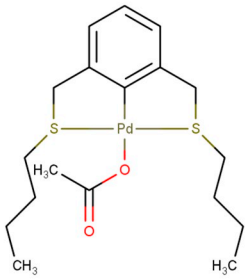
Epoxy-alpha-lapachone



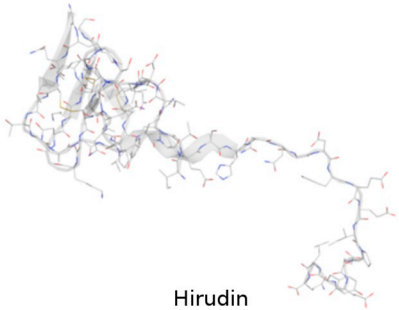
s9
(N-acylated trans-aziridine-2,3-dicarboxylate)



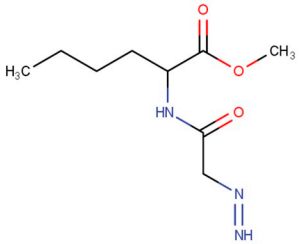
1c
(2-hydroxy-4-O-(3,3-dimethyl)-allylbenzophenone)



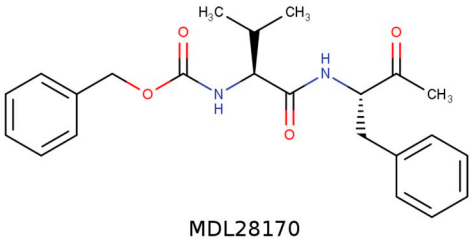
11
(aceto[2,6-bis[(methylthio-kS)methyl]phenyl-kC]-(SP-4-3)PdII)



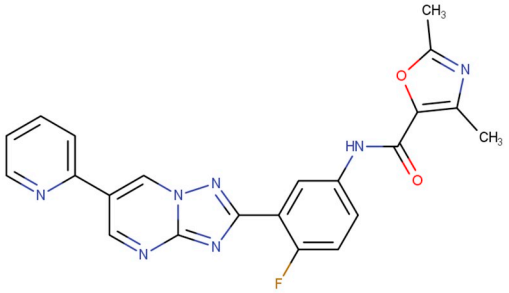
Hirudin
(PDB code 4HTC, Rydel et al., 1991)



DAN
(Diazoacetyl-DL-norleucine methyl ester)



MDL28170
(Z--Val-Phe-CHO)



GNF6702

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Fig. 4. Chemical structures of some protease inhibitors evaluated against *Leishmania* sp.

B) and *L. mexicana* (cathepsin L) without affecting the cathepsins B and L of the mammalian host. This compound exhibited IC_{50} of 37.4 μM and 2.3 μM for promastigotes and amastigotes of *L. major*, respectively (Table 2) [102].

Almeida and colleagues evaluated the effect of benzophenone derivatives on cysteine proteases of parasites (rCPB2.8, rCPB3.0 and cruzain) and on intracellular amastigotes of *L. amazonensis*. The most effective benzophenone derivative (named 1c, Fig. 4) showed a significant inhibitory effect on these enzymes and exhibited good activity against amastigote forms (IC_{50} = 74.4 μM). Furthermore, this compound had low toxicity on host macrophages (Table 2) [103]. Interestingly, Fricker and colleagues evaluated the effect of metal-containing compounds on protozoan cysteine proteases and against parasites. Compound 11 (Fig. 4), a palladium complex, caused 100% of *L. major*, *L. mexicana* and *L. donovani* promastigotes mortality, at 10 μM to 1 μM, after 4 to 8 days of culture. It was proved that this compound is a potent inhibitor of parasite cysteine proteases, such as cruzain from *T. cruzi* and CPB from *L. major* [93]. Massai and colleagues evaluated the effect of different gold complexes on CPB2.8ΔCTE, a cysteine protease of *L. mexicana,* and found promising results. Among them, the best gold complex (AuL12) showed a concentration of 5.65 μM for inhibition of 50% of this protease. Interestingly, these gold complexes also presented a significant effect on *L. infantum* amastigotes, with IC_{50} below 3 μM. However, a correlation between the activity against *L. mexicana* protease and the effect on this parasite was not observed. These results suggest that *Leishmania* cysteine proteases are important cellular targets of gold complexes, but they are not the only ones, since these metal compounds may interfere on several cellular pathways which are essential for parasite survival [104].

Calpains are calcium-dependent cysteine proteases [105,106] which, in *T. brucei,* have important roles on morphology, flagellum formation and parasite growth [107,108]. With the elucidation of trypanosomatids genome, a total of 18 calpain-like proteins in *T. brucei*, 24 in *T. cruzi* and 27 in *L. major* [109,110] were disclosed. D'Avila-Levy and colleagues showed that compound MDL 28170 (*Z*-Val-Phe-CHO, Fig. 4), a potent dipeptidyl aldehyde calpain inhibitor, caused significant growth inhibition of *L. amazonensis* promastigotes, with IC_{50} = 23.3 μM, after 48 h of treatment (Table 2). Furthermore, in this same study, an anti-calpain antibody recognized a 80 kDa polypeptide, highlighting the possibility of the existence of calpains in *L. amazonensis* without homologues in mammalian [111]. Recently, Marinho and colleagues demonstrated that MDL 28170 induced apoptosis-like cellular death in *L. amazonensis* promastigotes, with depolarization of mitochondrial membrane, cellular rounding, formation of blebs in the plasma membrane, increase of promastigotes positive for annexin-V and DNA fragmentation [112].

3.1.3. Metalloproteases

Various human pathogens produce metalloproteases with a wide variety of pathological effects. Zinc is an essential metal ion for the catalytic activity of these proteases. Metalloprotease activity has been described in several parasites of the Trypanosomatidae family, like *L. amazonensis*, *L. braziliensis*, *L. chagasi*, *L. donovani* and *L. tropica* [113]. This protease is expressed in all *Leishmania* species and in both parasite stages, although the expression level in amastigotes is < 1% of what was found in promastigotes [114–117].

The major surface protease of *Leishmania* parasites is the glycoprotein 63 (GP63) or leishmanolysin, a zinc-dependent metalloprotease [115,117,118]. Many functions can be attributed to GP63, such as parasite nutrition in insect vectors [117], infection in host cells [119,120], enhancement of phagocytosis by host cells, survival within macrophages and enhanced resistance to complement-mediated lysis [118,121]. Corradin and colleagues showed that GP63 from *L. major* degrades substances present in macrophages, impairing the activation of these host cells [122]. Furthermore, Lieke and colleagues showed that GP63 of *L. major* promastigotes binds to human natural killer (NK) cells and inhibits their proliferation, impairing their effector role in control and resistance against *Leishmania* infections [118]. In view of the important role of GP63 in survival and virulence of parasites, it is indispensable to discover new inhibitors of this protease.

In this context, Khan and colleagues carried out a virtual screening of many protease inhibitors against GP63 metalloprotease. Different compounds showed greater binding affinities to GP63, such as tipranavir, itraconazole and atazanavir, with 50% of protease inhibition from *L. major* estimated as 0.05, 2.2 and 0.011 μM, respectively. However, the best compound was hirudin (Fig. 4), a substance with anticoagulant properties which presented the lowest binding affinity (−13.7 Kcal/mol) and with 50% of protease inhibition estimated as 0.0068 μM. Interestingly, *in vitro* assays disclosed that hirudin is active against promastigotes and axenic amastigotes of *L. tropica*, with IC_{50} values of 0.60 and 0.43 μg/mL, respectively (Table 2). This compound caused cell death by apoptosis and increased the plasma membrane permeability. Moreover, hirudin did not show adverse effects on human macrophages (CC_{50} = 860.11 μg/mL) (Table 2) [123].

3.1.4. Aspartic proteases

Enzymes from the group of aspartic proteases have been established as therapeutic targets for different human diseases [124]. In *L. amazonensis* and *L. mexicana,* lytic activity capable of degrading aspartic protease substrates has been detected. This activity can be specifically inhibited by pepstatin A and diazo-acetylnorleucinemethylester (DAN, Fig. 4) [124–126]. In *L. mexicana*, the aspartic protease activity possibly plays a role in cell proliferation. Interestingly, DAN was effective against *L. mexicana* promastigotes, with an IC_{50} of 22 μM after 72 h of treatment (Table 2), and this compound caused rounding of the cell body and inhibited cell division, showing parasites with at least 2 nuclei [126].

Leishmania parasites, particularly the species that cause visceral leishmaniasis, such as *L. infantum* and *L. donovani,* behave as opportunistic pathogens in patients with HIV [127]. Furthermore, these parasites are able to potentiate virus replication [128]. In face of this fact, inhibitors of HIV aspartyl proteases are frequently tested against *Leishmania* species. Valdivieso and colleagues evaluated two inhibitors of HIV aspartic proteases, Nelfinavir and Saquinavir, on several *Leishmania* species. Both caused inhibition of *L. infantum*, *L. donovani*, *L. mexicana*, *L. amazonensis*, *L. braziliensis* and *L. major* promastigotes proliferation, with Nelfinavir being more effective than Saquinavir. These compounds inhibited the aspartic protease activity of *L. infantum* and *L. mexicana*. These two aspartic protease inhibitors also reduced the percentage of infected macrophages with *L. mexicana* and *L. infantum* amastigotes and the number of amastigotes per macrophage. Furthermore, Nelfinavir and Saquinavir decreased HIV/*Leishmania* coinfection. This leishmanicidal effect was associated with the inhibition of cell division [129].

Santos and colleagues also evaluated the effect of Nelfinavir in *Leishmania*. This compound inhibited > 90% of the *in vitro* cell proliferation of *L. amazonensis*, *L. braziliensis, L. infantum* and *L. donovani* promastigotes and about 50% of *L. major* at 25 μM after 72 h. Nelfinavir was also able to inhibit the proliferation of *L. infantum* strains isolated from patients co-infected with HIV. Moreover, this HIV protease inhibitor significantly reduced the aspartic protease activity of *L. amazonensis*, *L. braziliensis, L. infantum*, *L. major*, *L. donovani* and *L. infantum* isolated from patients co-infected with HIV [130]. Kumar and colleagues verified that Nelfinavir induces cell death by apoptosis in axenic amastigotes of *Leishmania* by DNA fragmentation and cell accumulation in the sub G0/G1 phase of the cell cycle. This effect was mediated by oxidative stress and occurred independently of caspases [131].

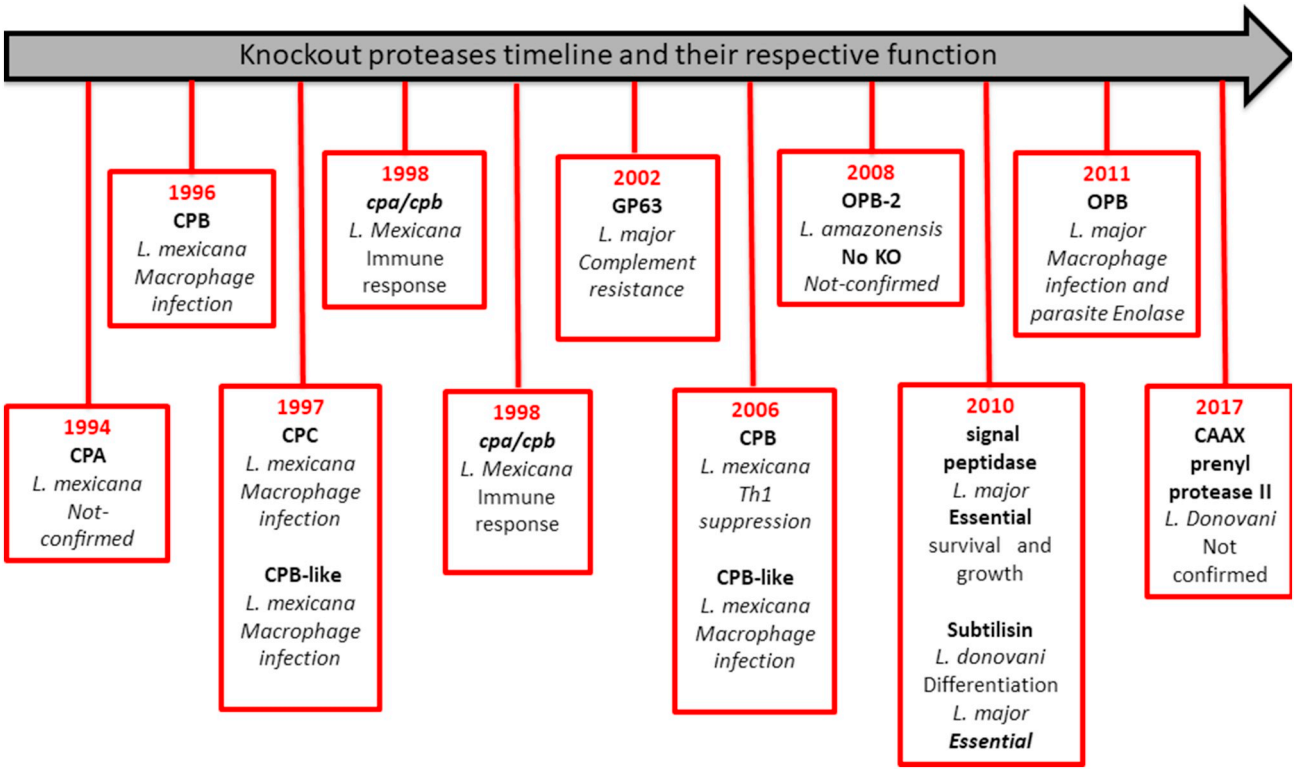

Fig. 5. *Leishmania* proteases timeline.

3.1.5. Proteasomes

The proteasome is a large non-lysosomal protease complex [132], located in the nucleus and cytosol of eukaryotic cells [133,134]. In protozoa, the proteasome has important roles in cell differentiation and replication [131]. Differences between mammal and parasite proteasomes have been observed, which suggests that protozoan proteasomes could be considered a chemotherapeutic target in different parasitic diseases [135].

The proteasome of *L. mexicana* was purified and characterized by Robertson, which has been shown to be a complex structure. Proteasome inhibitors, such as benzoyloxycarbonyl-leucyl-leucyl-leucinal (MG-132) and *N*-acetyl-leucylleucyl-norleucinal (ALLN) affected the growth of *L. mexicana* promastigotes and amastigotes [136]. In *L. infantum*, the proteasome is involved in the replication of promastigotes and in the survival of amastigotes in the host cell [133]. Furthermore, it has been demonstrated that *Leishmania* infection blocks the JAK2/STAT1 pathway in macrophages, which prevents the cell from responding to IFN-Ɣ and allows the survival and propagation of parasites inside these cells. This occurs because the protein tyrosine phosphatase, SHP-1, inactive JAK2 and STAT1 are degraded by *Leishmania* proteasomes, showing that the proteasomes contribute to subvert the host immune response by *Leishmania*, favoring the survival of these parasites [137].

Micale and colleagues already reported that gold complexes can be potent inhibitors of proteasomes of cancer cells [138]. Massai and colleagues verified that the gold complex ($Au(NHC)_2PF_6$), at 20 μM, caused about 69% inhibition of proteasome CT-L [104]. Savoia and colleagues evaluated the effect of indinavir and saquinavir in *L. major* and *L. infantum*. These compounds are known as HIV protease inhibitors and reduce proteasome activity [139]. Interestingly, indinavir and saquinavir showed activity against *L. major,* which was more sensitive than *L. infantum*. The IC_{50} in *L. major* promastigotes was 8.3 and 7 μM for indinavir and saquinavir, respectively (Table 2). The effect of these compounds in *Leishmania* is possibly related to their effects on the proteasome activity of the parasite [139].

Khare and colleagues identified, among 3 million compounds, an azabenzoxazole (GNF5343) with significant effect against trypanosomatids, including *L. donovani* intracellular amastigotes (IC_{50} = 7.3 μM) (Table 2). This compound was modified yielding azabenzoxazole compounds that were more potent against amastigote forms of *L. donovani*, such as GNF2636 (IC_{50} = 350 nM) and GNF3849 (IC_{50} = 71 nM). The modified structure of GNF5343 produced GNF6702 (Fig. 4), an azabenzoxazole compound that is more effective against *L. donovani* amastigotes (IC_{50} of 18 nM) and less toxic to host macrophages (Table 2). GNF6702 was also very active against *T. cruzi* and *T. brucei*. The molecular target of these compounds was the proteasome, which GNF6702 showed significant inhibition of activity of *T. cruzi* proteasome, without inhibition of the human proteasome. Interestingly, this compound also showed a good *in vivo* effect against *L. donovani* and *L. major*. After 8 days of treatment with GNF6702, a reduction of 99.8% of parasite burden was achieved in the liver of mice infected with *L. donovani,* and a treatment for 7 days of mice infected with *L. major* resulted in a significant decrease in the parasite burden in footpads. This compound was also effective in a murine model of Chagas disease and sleeping sickness [140].

4. Gene disruption to validate proteases as a drug target

Before starting virtual screening, it is necessary to know if the gene in question is essential for parasite survival. Target validation of parasites has been performed through homologous recombination for a long time [141]. When the gene is considered essential, the parasite does not develop. To better understand the role of proteases in the search for new therapeutic targets, studies with deficient *Leishmania* strains were carried out (Fig. 5). Cysteine proteases, such as CPA [142], CPB [143,144] and double KO (CPA/CPB) [145,146], metacaspase [147] and GPI8 [148], metalloproteases such as GP63 [149] and CAAX prenyl protease II [150] are not essential. We can notice that the only essential protease family comprises the serine proteases, such as signal peptidase [85] and subtilisin for *L. major* [84].

The importance of serine proteases as a virulence factor was suggested in *Leishmania* [72] and these enzymes were studied in *Leishmania*, mainly in the characterization of proteasomes [136].

Several researchers successfully used the deletion strategy by homologous gene recombination to investigate the role of serine proteases in recent years, and it was not different with SPase. SPase is a transmembrane protein belonging to Clan SF family, S26A subfamily. To study its function in *L. major*, several transfection attempts to generate a deficient homozygous lineage were performed. After several unsuccessful attempts, only one heterozygous lineage was viable, suggesting the great importance of this gene for the survival of this species. In addition, the heterozygous lineage was unable to induce lesions in BALB/c mice, which are susceptible to *L. major* infection [85].

Identification and characterization of subtilisin were performed. In this study, *L. major* and *L. donovani* subtilisin-deficient parasites were developed, but it was only possible to generate homozygotes in *L. donovani*, showing that this is an essential gene in *L. major*. Differentiation and electron microscopy studies enabled the detection of a defect of this ability in deficient lineages. When subject to the appropriate conditions to differentiate into amastigotes, the promastigotes of the deficient lineage did not form aggregates of cells typically seen in cultures of axenic amastigotes. Additionally, they presented morphology very different from WT *Leishmania* [85].

OPBs are other serine proteases which have been described as promising therapeutic targets. These enzymes are endopeptidases belonging to the prolyl oligopeptidases family, S9A, which is characteristic of the beta propeller domain. This domain restricts enzyme specificity to peptides smaller than 3 kDa. In the study of oligopeptidases from *L. amazonensis,* our group cloned and sequenced the first OPB-like (or OPB2) from *L. amazonensis*. Furthermore, the presence of OPB was observed in all phases of the parasite life cycle, as well as the conservation between the catalytic domain and the β propeller domain, when compared by molecular modeling [79,80]. A study in *L. major* showed that the lack of OPB in genetically modified parasites resulted in a decrease of infectivity *in vitro*, but did not significantly modify the *in vivo* infection [83]. In *L. donovani*, OPB deficient parasites were also less able to infect and survive within macrophages [69]. Observing the parasites deficient in OPB, the hypothesis of compensating the lack of OPB by the OPB2 gene was raised [83].

It is important to note that the determination of essential genes by using only homologous recombination could lead to mistakes. For example, metacaspase was considered essential by Ambit and coworkers at 2008. However, with the advancement of technology, it was possible to knockout the gene [147]. Nowadays, the use of conditional gene depletion with DiCRE is required to determine target validation [151,152].

The knowledge about the importance of each protease in *Leishmania* enables the use of modern strategies to discover novel compounds with satisfactory leishmanicidal effect and contribute with improvements in the treatment of leishmaniasis. Two of these strategies will be addressed in this work: molecular modeling and nanotechnology.

5. Molecular modelling as a useful tool for antileishmanial drug development based on proteases

Computer Aided Drug Design (CADD) has been widely used to reduce the number of compounds required to experimental evaluations [153]. The best predicted compounds regarding potency and ADMET parameters could be then subjected to further experimental testing. It reduces cost, workload and the number of animals used in the assays.

Structure-based drug design (SBDD) employs structural information about the target, based on the hypothesis that favorable interactions between molecules and specific proteins can exert a biological effect [153]. The target structure is usually determined by X- ray crystallography or NMR techniques. In the absence of experimentally determined structures, comparative modeling techniques can be applied to predict the protein 3D structure. Several studies based on comparative modeling of target proteins have been reported to *Leishmania* proteases [79,154–158].

In studies conducted by de Matos Guedes and colleagues, OPB from *L. amazonensis* were cloned and sequenced [79]. The authors used the homology modeling technique to obtain the 3D structure of *L. amazonensis* OPB and compared it with prolyl oligopeptidases from other organisms. The charge on the surface of *L. amazonensis* OPB is mainly negative, in contrast to the surface of prolyl oligopeptidase from porcine brain. However, the salt bridge formed by Asp134 and Arg664 is conserved. The differences and similarities between S1 and S2 subsites were also investigated. The S1 subsite of OPB from *L. amazonensis* is negatively charged due to the presence of two glutamic acids. Besides that, the S2 subsite of *Leishmania* OPB is also different from bacterial and trypanosomes oligopeptidases. Since OPB showed preference for the cleavage of dibasic amino acids, the authors identified Asp174, Glu205, Glu247 (S1) and Asp504 (S2) as important residues for substrate recognition by ionic interaction with basic residues.

Molecular docking is a computational method usually explored in drug discovery to analyze the probable binding mode and affinity of a small molecule within the target binding site [159]. It is also used to perform a virtual structure-based screening to evaluate larger compound libraries and find potential ligands. In 2017, *L. amazonensis* OPB model was rebuilt by our research group [157] based on the 3D structure of *L. major* OPB deposited on PDB [160]. *L. amazonensis* OPB2 was also built and both enzymes were used to perform a virtual screening based on the binding site structure to identify possible dual inhibitors. Important residues were indicated to both enzymes, such as His688, Glu612, Pro607, Arg655 and Tyr490 to OPB and Tyr537, Glu539, Ser614, Ala615, Phe641, Leu655, Glu659 and Arg704 to OPB2. The best scored compounds were also evaluated by ADMET calculations and showed good results. Four molecules were identified as important inhibitors of both OPB and OPB2, acting simultaneously on the two enzymes, which showed better results than the antipain (Fig. 6) and drugs currently used in the treatment of leishmaniasis [157].

Souza-Silva and colleagues investigated effects of epoxy-alpha-lapachone in serine proteases. The authors analyzed the binding mode of epoxy-alpha-lapachone in OPB from *L. amazonensis* by computational methods. The homology modeling technique was performed to build the protein 3D structure, followed by compound docking. The study was able to suggest that the complex conserves the binding profile of antipain, maintaining the hydrogen bonds at S1 and hydrophobic interactions. It also indicated important residues to form hydrogen bonds (Ser577, Ala578, and Tyr496) and hydrophobic interactions (Phe698, Arg576, Ile501, and Leu617) [90].

Goyal and colleagues also studied OPB [161]. The authors used naturally occurring compounds from ZINC database to carry out a virtual screening on *L. major* OPB, followed by molecular dynamic simulation (MDS) and ADMET properties calculation of the best scored compounds. Two compounds stood out, COP (ZINC code 67902758) and TOA (ZINC code 70672673). Although important changes in pattern interactions were observed after MDS, residues Tyr499, Ser577, Arg664, His697, Phe698 and Ala700 were still involved in COP-OPB complex interaction and residues Tyr499, Ser577, Phe603, Thr609, Ile615, Pro616, Leu617, Gly620, Glu621 and Val665 in TOA-OPB complex interaction. The two compounds also showed good absorption power and low toxicity predictions.

In 2016, Singh and co-workers built the 3D structure of CAAX prenyl protease I and II of *L. donovani* by comparative modeling [156]. The models were validated and the protein active sites were predicted. Structural differences were highlighted, which suggest distinct biological activities. The models were further used to perform docking, considering induced fit, of farnesyl transferase inhibitors and peptidyl (acyloxy) methyl ketones, virtual screening, binding free energy calculations, ADME properties prediction and MDS. Known ligands show better docking scores with CAAX prenyl protease I than CAAX prenyl

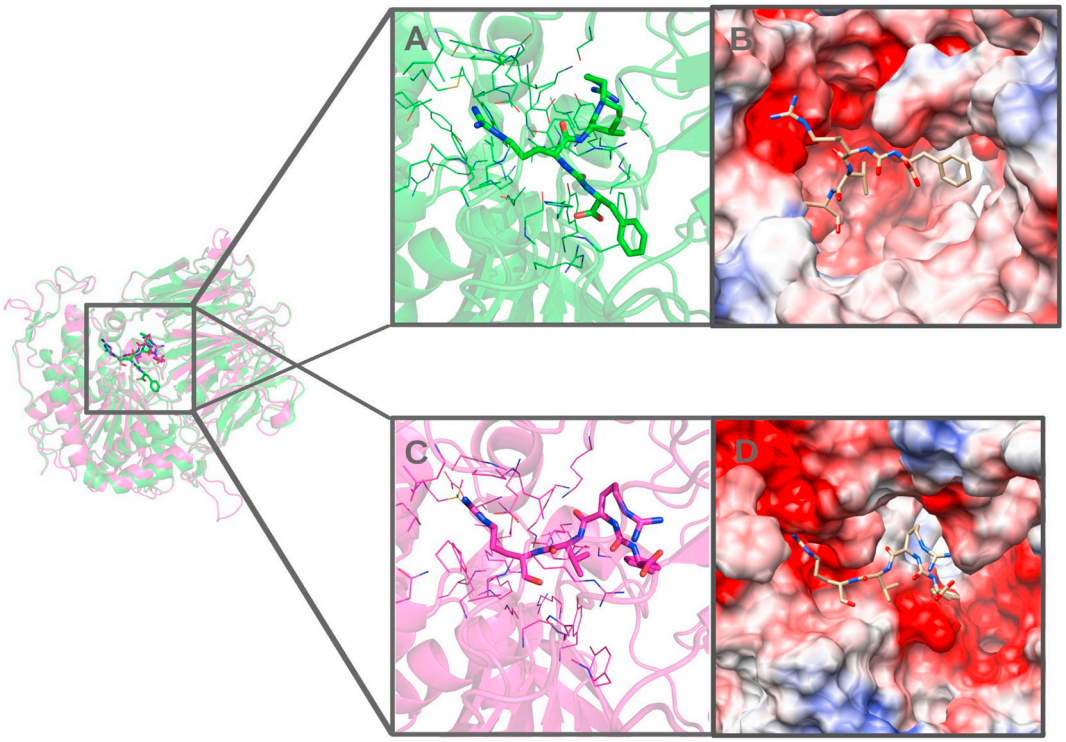

Fig. 6. Molecular modelling as a tool for drug development against proteases. Proposed binding mode of antipain in OPB (in pink, A and B) and OPB2 (in green, C and D) from *L. amazonensis*, *obtained* by molecular docking [SODERO, 2016]. Residues around 4Å from antipain are represented as lines. The solvent-accessible surface of the proteins is colored by electrostatic potential. Blue corresponds to positive potential and red to negative potential. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protease II, as evidenced by their binding energy calculations. The authors also performed a virtual screening considering molecules from three databases. Interestingly, the screened compounds presented better docking scores than known ligands. The best complexes of both proteins remained stable through MDS. Physicochemical and pharmacokinetic evaluations also corroborate that these ligands can be promising CAAX prenyl protease I and II inhibitors.

A study performed by Scala and colleagues generated a structural model of active mature *L. mexicana* cysteine protease isoform without the C-terminal extension (CPB2.8DCTE) by homology modeling. The authors performed the non-covalent and covalent docking of an anhydride-based compound, which is a potent and highly selective CPB inhibitor, followed by MDS. The results of the non-covalent redocking showed that the inhibitor binds to the enzyme, exposing both carbonyl and carboxyl moieties to the nucleophilic carbon residue involved in the formation of the covalent bond. The covalent docking, which used as starting conformation the best non-covalent docked pose, suggested that the inhibitor is deeply embedded in the active site with both carboxylic moieties forming hydrogen bonds with Gln19, Cys25, and Trp185. The results from experimental and computational calculations indicate that the ligand first acts as a non-covalent inhibitor, and then it covalently binds to the protein [155].

Schröder and colleagues also used a 3D model to perform a covalent docking [154]. The homology modeling technique was applied to build the mature *L. mexicana* cysteine protease, CPB2.8ΔCTE. It was noted by comparing the *L. mexicana* protein and bovine cathepsin B (BtCatB) that there are different residues in the S2 subsite, which can be used to find selective inhibitors, although some similarities in S1 subsite were also found. Through high throughput screening, the authors identified four lead structures as selective inhibitors of CPB2.8ΔCTE. Compound CP229988 showed good activity and high specificity. It was observed, from covalent docking calculations, that this inhibitor binds differently in CPB2.8ΔCTE and BtCatB. CP229988 preferentially binds to S1 and S2 subsites of CPB2.8ΔCTE, but presents a poor docking score when docked in the BtCatB binding site. The differences between the binding interactions of both proteins indicated additional compound optimization to develop potent and selective drug candidates.

Shaukat and co-workers designed, synthetized and tested a series of benzimidazole derivatives. The authors performed the molecular docking of benzimidazole derivatives on *L. major* leishmanolysin GP63 to evaluate the derivative binding modes and to compare them with amphotericin B. They suggest that Glu265, Glu220 and Zn produce a hydrogen bonding network. The aromatic interactions are indicated as being important to GP63 inhibition, especially to residues His264 and His268. The compounds also showed favorable pharmacokinetics parameters, indicating that they may be potential drug candidates [162]. The work of Gomes and co-workers investigated 32 previously synthesized chalcones and chalcone-like compounds to evaluate the antileishmanial activity. The compounds were submitted to target fishing by employing pharmacophore modeling. The structures of 7 selected targets were built by homology modeling and submitted to molecular docking calculations. Docking results indicated 9 compounds as possible active and 3 as potentially inactive compounds, and the compounds (LabMol-65, LabMol-72, LabMol-73, and LabMol-92) might interact with the cysteine protease procathepsin L. Thus, the authors cite the hydrophobic pocket as being important for the interaction with procathepsin L and having a crucial role in the interaction of Trp151 with chalcone. *In vitro* assays showed a reasonably high activity and low cytotoxicity of LabMol-65 and Labmol-73, which make them promising drug candidates against leishmaniasis [158].

Computational methods have been established as important tools to optimize lead compounds, helping in the identification of more selective and safe new drug candidates against leishmaniasis.

6. Nanotechnology in the development of treatments

Nanosystems consist on the development of drug delivery systems with at least one of their dimensions in the nanometric scale. These systems play a key role in the development of innovative therapeutic strategies due to their versatility and ability to enhance pharmacokinetic properties, improve bioavailability, reduce toxicity and protect drugs against physicochemical and biological degradations. Interest in the development of new leishmaniasis treatments based on nanosystems has grown significantly in recent years, as can be seen in recent reviews in the area [163–165]. Despite this, until now, only liposomal amphotericin B was approved for the treatment of human leishmaniasis. Further studies and clinical trials are needed to explore the advantages of nanosystems in the treatment of leishmaniasis. Here, we discuss some strategies used to improve the properties of protease inhibitors through the use of nanosystems, which could be also used to increase the antileishmanial activity of these molecules.

Aspartic protease inhibitors already used in current AIDS chemotherapy (*e.g.* amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir) emerged as promising candidates for treating leishmaniasis, although these compounds exhibit incomplete absorption and rapid systemic clearance [124]. To overcome this challenge, some research groups are exploring the use of nanosystems to improve the bioavailability, aqueous solubility and permeability of these compounds. In this sense, the use of saquinavir nanocrystals with a rod-like shape (200 nm) was able to significantly improve its oral absorption [166]. In another study, the use of saquinavir in PEGylated solid lipid nanoparticles (275 nm), that increases the transport capacity of the drug through intestinal epithelial cells, increasing its relative bioavailability, also reduced its cytotoxicity [167]. Beloqui and colleagues showed that the size and surfactant content in saquinavir-loaded nanostructured lipid carriers (LNC) were essential to change the nanoparticles transcytosis mechanism and to overcome the P-gp drug efflux. The modification of these critical physicochemical parameters caused a 3.5-fold increase of LNC transport across Caco-2 monolayers when compared with drug suspension [168].

Besides the drug bioavailability improvement, the use of nanosystems, such as nanogold particles conjugated with saquinavir, may also promote sustained drug release, and together, these characteristics increase the drug efficacy even with the reduction of the number of doses and consequent reduction of adverse effects [169]. In addition to saquinavir, the use of nanosystems, such as drug nanocrystals [170] and poly (lactic-*co*-glycolic acid) (PLGA) nanoparticles [171] was also carried out to improve permeability, the relative bioavailability and at least the oral absorption of nelfinavir.

The major challenge in leishmaniasis treatment is ensuring that the drug reaches the parasite within the macrophage phagolysosome. For this, high and multiple drug doses should be administered, which results in toxicity and resistance. In this context, nanotechnology has emerged as an interesting strategy to overcome this dilemma. Indeed, while molecules have difficulty to overcome biological barriers, colloidal nanosystems like liposomes and polymeric nanoparticles are readily internalized by cells of the mononuclear phagocyte system. Thus, the nanosystems are able to release the drug inside the macrophages and lead to an effective local concentration to kill the parasite, even with total dose reduction and consequently reduced toxicity [163]. A simplified scheme of the interaction of drug delivery nanosystems with *Leishmania*-infected macrophages is shown in Fig. 7. Furthermore, nanosystems still have the advantage of restricting the action of protease inhibitors to parasite enzymes, due to drug targeting to macrophages, reducing toxicity. In this sense, saquinavir-loaded chitosan carriers showed higher T-cells targeting efficiency (92%) and superior control of viral proliferation when compared with the soluble drug [172]. In another study, saquinavir-loaded poly (ethylene oxide)-modified poly (epsilon- caprolactone) (PEO–PCL) nanoparticles, showed the best uptake in THP-1 human monocyte/macrophage (Mo/Mac) cells in relation to the drug in aqueous solution [173].

Besides this, the nanosystem surface can be conjugated with biological compounds, such as an antibody, peptide or mannose, to improve the macrophage selectivity and reduce the toxicity to normal cells [174]. Nanoparticles can be produced to specifically target the immune system or to avoid them. Changes in their physicochemical properties, such as size, surface properties, shape and solubility, are directly correlated with the endocytic pathway adopted (clathrin/caveolar-mediated endocytosis, pinocytosis, phagocytosis or macropinocytosis) and clearance by macrophages [175]. In this context, Liptrott and colleagues demonstrated that solid drug nanoparticles loaded with lopinavir have the potential to mitigate the undesired immune effects of the drug in the AIDS treatment, since contrary to the free drug, the nanosystem was not able to activate the secretion of IL-1β and TNF-α by monocyte-derived macrophages [176].

Despite these different studies demonstrating the improvement of protease inhibitor properties with the use of nanosystems, none of them were evaluated with regard to their efficacy in the treatment of leishmaniasis. We only identified one study in the literature that demonstrates the improvement of the anti-amastigote activity in *L. amazonensis* and the safety in macrophages with the use of block copolymers based on nanoparticles loaded with 4-nitrobenzaldehyde-thiosemicarbazone, a promising inhibitor of cysteine protease [177]. However, additional studies should be carried out to evaluate the efficacy and safety of this system *in vivo*. Additionally, further efforts must be made to effectively use nanosystems as a strategy to improve the antileishmanial activity of protease inhibitors.

7. Perspectives

For drug development, target validation by molecular biology is an important step in the characterization of essential genes for *Leishmania*. In the past, homologous recombination was used; however, the process is very time-consuming. Presently, the development of the Crisp-Cas9 system for *Leishmania* allowed the improvement of identification and streamlines the finding of essential genes [141]. Besides this, the use of conditional gene deletion by DiCre is necessary to prove concepts to target validation [151,152].

The use of Systems Biology to determine the function, protein-protein interaction or cascade signaling network for each protease and the use of Chemical Systems Biology to determine the targets and effects of proteases inhibitors are essential to improve drug development [178–180]. Even though these approaches have already produced important advancements with direct impact on human health, the use of these approaches in neglected tropical diseases, such as leishmaniasis and proteases, needs to be deepened [181].

Despite of the high number of sequences from *Leishmania* deposited in databases, such as Genbank (nucleotides) [182] and Uniprot (proteins) [183], the Protein Data Bank [184] currently has only 444 entries, of which 95 are transferases. It reinforces that experimental methods to determine target 3D structures do not follow the number of known protein sequences. Thus, computational methods, such as comparative modeling, would be valuable. In order to predict protein models, homology modeling techniques are widely used, but they require at least one 3D structure of related proteins known. The sequence identity is also successfully related to the method [185]. Besides this, there are unique proteins whose folding is completely unknown. In these cases, *ab initio* modeling can be used and the structure is modeled only by sequence. Although many efforts have been made to obtain reliable models, the *ab initio* programs to predict protein models are still challenging.

The combination of High-Throughput Drug Screening with target/structure based drug screen. The use of High-Throughput Drug Screening is an alternative to find anti-leishmanial compounds. In this technology, molecules with the ability of controlling the parasite load directly in infected macrophages are searched using different chemical

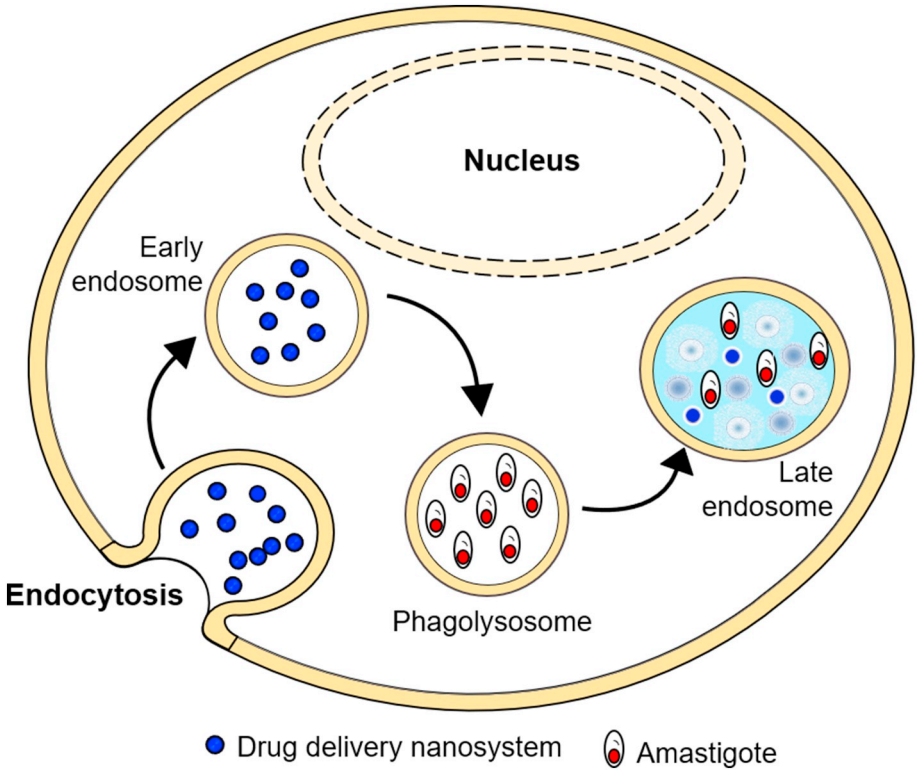

Fig. 7. Simplified scheme of endocytosis patterns of drug delivery nanosystems by *Leishmania*-infected macrophages. Following internalization, nanosystems are transported in vesicles (early endosome) that eventually merge with different endosomes and lysosomes, forming the late endosomes. In this process, the drug is released directly at the site of infection.

libraries. Several molecules (*e.g.*, 4000 compounds) were tested concomitantly using the platform [186]. The great advantage of this platform is the time decrease to test the drugs and the increase of chances to find molecules that would become drugs. In this pipeline, it was possible, after evaluating 400 molecules, to find two drug candidates. The analysis of several manuscripts using different platforms and *Leishmania* species allowed us to find drug candidates [187–189]. In this review, we suggest a different strategy based on the target and structure. However, we can combine both strategies. After finding a target, we express the protease and use High-Throughput to screen protease inhibitors. After finding better inhibitors, we perform drug testing using the High-Throughput drug screen platform. This strategy will open up the possibility of finding new peptidade inhibitors which could be drug candidates against leishmaniasis.

The development of new formulations using nanotechnology to develop protease inhibitors is an urgent need for leishmaniasis treatment. Independently of the use of a very specific inhibitor against parasite proteases, the development of a delivery system ensures that the drug is deposited inside phagolysosomes of macrophages, which will reduce toxicity, the greatest problem of chemotherapy in leishmaniasis.

8. Conclusion

Further studies involving clinical trials against leishmaniasis are needed. Many molecules are already designed and postulated as candidates for new drugs based on protease inhibitors. However, none of these molecules are being currently studied in clinical trials (www.dndi.org/2016/clinical-trials/clinical-trials-leish/; https://www.clinicaltrials.gov/). The study of protease inhibitors may lead to the development of novel antileishmanial molecules, and proteasome inhibitors would be the first targets for this purpose.

Abbreviations

AIDS	acquired immune deficiency syndrome
ADMET	pharmacokinetic parameters (absorption, distribution, metabolism, excretion and toxicity)
APCs	antigen-presenting cells
CADD	computer aided drug design
CL	cutaneous leishmaniasis
CPB	cysteine protease B
HIV	human immunodeficiency virus
MDS	molecular dynamic simulation
NK	natural killer
NO	nitric oxide
OPB	oligopeptidase B
ROS	reactive oxygen species
SBDD	structure-based drug design
SCCA	squamous cell carcinoma antigens
Spase	signal peptidase
VLPs	virus like particles
VL	visceral leishmaniasis
WT	wild type

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

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