

Why calpain inhibitors are interesting leading compounds to search for new therapeutic options to treat leishmaniasis?

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(Received 14 June 2016; revised 1 September 2016; accepted 26 September 2016; first published online 21 November 2016)

SUMMARY

Leishmaniasis is a neglected disease, which needs improvements in drug development, mainly due to the toxicity, parasite resistance and low compliance of patients to treatment. Therefore, the development of new chemotherapeutic compounds is an urgent need. This opinion article will briefly highlight the feasible use of calpain inhibitors as leading compounds to search for new therapeutic options to treat leishmaniasis. The milestone of this approach is to take advantage on the myriad of inhibitors developed against calpains, some of which are in advanced clinical trials. The deregulated activity of these enzymes is associated with several pathologies, such as strokes, diabetes and Parkinson's disease, to name a few. In *Leishmania*, calpain upregulation has been associated to drug resistance and virulence. Whereas the difficulties in developing new drugs for neglected diseases are more economical than biotechnological, repurposing approach with compounds already approved for clinical use by the regulatory agencies can be an interesting shortcut to a successful chemotherapeutic treatment for leishmaniasis.

Key words: Calpain inhibitors, leishmaniasis, repurpose approach, MDL28170.

INTRODUCTION

The Trypanosomatidae family, Kinetoplastea class, is composed of a large group of exclusively parasitic protozoa, some of which cause important diseases in humans (d'Avila-Levy *et al.* 2015). Altogether, about 37 million people worldwide are infected with *Trypanosoma brucei*, the aetiological agent of African sleeping sickness; *Trypanosoma cruzi*, the causative agent of Chagas' disease or American trypanosomiasis; and different species of the genus *Leishmania*, which are responsible for a wide spectrum of clinical manifestations known as cutaneous, mucocutaneous and visceral leishmaniasis (WHO, 2015). Since these diseases have been wiped out in the more developed parts of the world and persist only in the poorest, most marginalized communities and conflict areas, they are classified as neglected tropical diseases by the World Health Organization (WHO, 2015). Factors like malnutrition, weak immunity, illiteracy, lack of resources and environmental changes, as well as the migration of non-immune people to endemic areas, play important roles in the dynamic of these diseases (Desjeux, 2001; Alvar *et al.* 2006; Boelaert *et al.* 2010).

All the clinical manifestations collectively known as leishmaniasis comprise one of the most prevalent neglected diseases worldwide with more than 2 million new cases occurring annually and endangering around 350 million people in 98 countries in the tropics, sub-tropics and European Mediterranean area (WHO, 2015). Visceral leishmaniasis, also known as kala-azar, is the most severe one, and can be fatal if untreated. This clinical manifestation affects the vital organs of the body and is characterized by irregular bouts of fever, weight loss, anaemia, and swelling of the spleen and liver. If not fully healed, visceral leishmaniasis can progress to post-kalazar dermal leishmaniasis, which is characterized by a hypopigmented macular, maculopapular and nodular rash. Cutaneous leishmaniasis, the most common form of the disease, causes ulcers on exposed skin areas of the body, leading to disfigurement, permanent scars, social stigma and in some cases disability. However, this is usually a self-healing illness. Finally, mucocutaneous leishmaniasis consists of the most destructive form of the disease, since it causes partial or total mutilation of mucous membranes in the nose, mouth and throat. In almost all cases, it may cause serious deformities (WHO, 2010; Alvar *et al.* 2012).

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TREATMENT AND DRUG THERAPY

Despite the great advances in combating infectious diseases over the past century, the current therapy

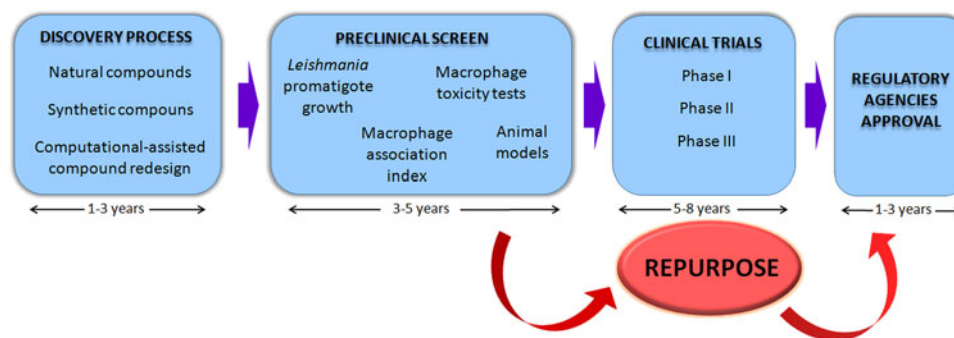


Fig. 1. Representation of the main steps necessary to the final drug approval and the shortcut represented by the repurpose approach.

to treat neglected diseases, like leishmaniasis, is extremely limited to a handful of drugs that suffer from unacceptable toxicity, high costs, difficulties of administration and increasing treatment failures, since resistance to these compounds has become a severe problem (Cavalli and Bolognesi, 2009; Wilkinson and Kelly, 2009; Boelaert *et al.* 2010). In view of this scenario, the development of new drugs is an urgent need, which has led to the investigation of several compounds chosen empirically, or through studies that identify promising metabolic targets to the rational drug design and selection (De Menezes *et al.* 2015).

The process of drug development is time consuming, laborious and expensive. On average, a drug is developed in 15–17 years, from the discovery process to pharmacological regulatory agencies approval. Before the availability for doctors to prescribe, millions are spent, starting from the discovery process that involves screening chemically diverse compounds (synthetic or natural sources), computational-assisted compound redesign, pre-clinical testing in cellular and animal models, to clinical trials and then the final approval (Fig. 1). Considering the neglected diseases, the reality is quite different, since the link with poverty results in low investment in rational drug development. A high number of patents are dead due to the failure of payment of maintenance cost, or revoked for not meeting patentability requirements, which seems to be an indication of the difficulties faced by research institutes and universities for disclosed compounds to reach the final stages of innovation and entering into the market (Machado-Silva *et al.* 2015). Consequently, governmental action is crucial to guarantee innovation and patient care and treatment. Therefore, considering that the challenges for the introduction of new compounds to treat neglected diseases are more economical than biological, repurpose drugs have potential benefits, such as, reducing the costs during discovery and development, preclinical laboratory tests and clinical phases (Andrews *et al.* 2014; Kwok and Koenigbauer, 2015) (Fig. 1).

CALPAIN INHIBITORS

Economically, drug-repurposing strategies have the potential to facilitate an effective drug development. As the cost associated with discovering and developing a new chemical entities may be around US\$ 800 million and may take one to two decades, repurpose drugs have already proven to have considerable advantages (Ashburn and Thor, 2004; Taylor and Wainwright, 2005; Nwaka and Hudson, 2006; Andrews *et al.* 2014) (Fig. 1). Taking into consideration this last possibility, a family of neutral calcium-dependent cysteine peptidases, the calpains, calls attention because a huge effort has been made to develop means of identifying selective inhibitors (Carragher, 2006; Donkor, 2015). These enzymes are involved in a variety of calcium-regulated cellular processes, such as signal transduction, cytoskeleton remodelling, cellular proliferation and differentiation, sex determination, membrane fusion, environmental regulated processes and apoptosis. Besides their physiological roles, calpains unregulated activity in humans is implicated in several pathophysiological processes, such as, aging, muscular dystrophy, multiple sclerosis, cataract, arthritis, cancer, strokes, diabetes and neurological disorders (Alzheimer's, Huntington's and Parkinson's diseases). It has been advocated that the specific inhibition of calpains under these condition can treat these pathologies. In addition to these pathologies, calpain unregulated activity also plays a crucial role in neuron death in traumatic spinal cord injury, and its specific inhibition can prevent apoptosis and restore transcription of proteolipid protein and myelin basic protein genes, which indicates the therapeutic efficacy of calpain inhibitors to rescue or prevent permanent disability. Therefore, prompt inhibition of calpains, if undertaken early enough after injury, could significantly spare many neurons (Huang and Wang, 2001; Battaglia *et al.* 2003; Saez *et al.* 2006; Zhang *et al.* 2015). To treat some of these pathologies, in the last 4 years, at least 52 calpain inhibitors were developed and screened (Donkor, 2015). Out of these, one is under phase I clinical trial to treat Alzheimer disease, and another compound presented promising

results in animal models to the treatment of cataracts and clinical trials will be performed by Calpain Therapeutics Co. (Table 1; Fig. 2). Up to date, calpain inhibitors display a wide range of potency, but low specificity, inhibiting also other cysteine and serine peptidases or even the proteasome (Low *et al.* 2016). Therefore, future efforts in the development of calpain inhibitors should not only concentrate on potency and selectivity of the inhibitors for calpain compared with other proteases but should also focus on achieving significant calpain isoform selectivity (Donkor, 2011).

Detailed studies on the kinetics and mechanism of action of calpain inhibitors allowed to classify them as: allosteric effectors, mechanism-based, tight binding, slow binding, affinity labels, suicide substrates, transition state analogues and dead-end inhibitors (Otto and Schirmeister, 1997; Vicik *et al.* 2006). Allosteric calpain inhibitors are known not to target the active site but most likely interact with allosteric sites, which are involved in catalysis and activation; as a result, these molecules may provide more therapeutic benefit than peptide inhibitors. An example is α -mercaptoacrylate PD150606, a potent and selective inhibitor of calpain-1 (Wang *et al.* 1996).

Active site-directed inhibitors, as the name suggests, interact directly with the active site. These compounds are usually peptidomimetics of calpain substrates, composed of modified amino acids that are recognized by the enzyme, and can be either reversible or irreversible inhibitors (Angelastro *et al.* 1990). Examples of the former include the synthetic compounds aldehydes, α -ketoheterocycles, and α -ketocarboxyls (Kawasaki *et al.* 1989; Tao *et al.* 1998), and natural compounds such as leupeptin (Mehdi 1991). Unfortunately, these inhibitors lack specificity, and attempt to improve them lead to loss of potency; additional problems to be surpassed are membrane permeability and solubility (Low *et al.* 2016).

Calpastatin is the natural endogenous inhibitor of calpains, and it is highly specific; its specificity is determined by the simultaneous binding of three calpastatin subdomains to both subunits of heterodimeric calpains (Battaglia *et al.* 2003; Carragher, 2006). Considering that the entire protein poorly penetrates the cells, fusion proteins containing a calpastatin peptide and a signal sequence capable of delivering the fusion protein into the cells have been produced and patented (reviewed by Donkor, 2015). Also, taking into consideration the mode of action of calpastatin, i.e. a β -turn loop within calpastatin forms a broad interaction around the active site cysteine that inhibits the enzyme, a library of peptidic compounds was constructed and are under test (Low *et al.* 2016). There are some promising results on K_i with a 1000-fold

selectivity for calpain compared with cathepsin L (Jiao *et al.* 2010).

CALPAINS IN TRYPANOSOMATIDS

Although calpains are well described in mammalian cells as well as its physiological roles and involvement in pathological disorders, in trypanosomatids, the picture is different. Calpains have a wide variety of domains in addition to the peptidase domain, such as calcium binding and penta-EF-hand domains. The calpain superfamily is divided into several subfamilies according to the structures of these additional domains. Since the mammalian conventional calpain catalytic subunits are the reference point for calpain structure, calpains having a similar domain structure are called 'classical' calpains in contrast to 'non-classical' ones that may lack one or all of these domains, including the peptidase domain. Non-classical calpain peptidase domains have amino acid identities with each other ranging from <25% to >75%, and they may have other functional domains in aleatory regions of the protein (Sorimachi *et al.* 2011). In addition, several of these non-classical calpains have alterations in the catalytic triad leading to proteins devoid of proteolytic activity.

A survey on trypanosomatids genome revealed a total of 18 calpain-like sequences in *T. brucei*, 24 in *T. cruzi* and 27 in *Leishmania major* (Ersfeld *et al.* 2005). Among these, some proteins present the catalytic triad conserved, which supports the idea that calpains may have proteolytic activity in trypanosomatids. However, these enzymes are tricky to detect biochemically, and may be readily hydrolyzed by other abundant peptidases. In this sense, calpain proteolytic activity was never demonstrated in *T. cruzi* or *Leishmania* spp. However, in *Angomonas deanei* (formerly *Crithidia deanei*), a monoxenic trypanosomatid, a proteolytically active cysteine peptidase was purified and presented several biochemical characteristics of calpains, such as neutral pH and loss of activity upon ions chelation, which was recovered after calcium restoration. Although the amino acid sequence of the purified protein was not demonstrated, the protein cross reacted with antibodies raised against an atypical calpain from *Drosophila melanogaster* (d'Avila-Levy *et al.* 2003). Also, two reports suggest the presence of a calcium-dependent cysteine peptidase in *Leishmania donovani*, but the molecular identity of the enzyme was also not assessed (Bhattacharya, Dey & Datta 1993; Dey *et al.* 2006). Therefore, more efforts should be directed to ascertain whether calpains are proteolytically active in trypanosomatids.

Considering the high number of genes and sequence diversity of calpains in trypanosomatids, it is not an easy task to completely characterize this protein family and to assess its functions. Up to

Table 1. A non-comprehensive list of available calpain inhibitors and its potentiality for clinical use, for an extensive list refer to Donkor (2015).

Inhibitors name, Alias	Most important structures	Commercial availability	Notes
A-705253, ABT-957	(<i>N</i> -(1-benzyl-2-carbamoyl-2-oxoethyl)-2-[<i>E</i> -2-(4-diethylaminomethylphenyl) ethen-1-yl]benzamide	Abbvie Inc.	In Phase I clinical trial to treat Alzheimer's Disease (ClinicalTrials.gov Identifier: NCT0222073)
CAT811	((7 <i>S</i> ,10 <i>S</i> ,13 <i>S</i>)-7-Formyl-10-isobutyl-9,12-dioxo-2-oxa-8,11-diaza-bicyclo[13·2·2]nonadeca-1(18),15(19),16-trien-13-yl)-carbamic Acid Benzyl Ester	Calpain Therapeutics Pty Ltd.	Safety and efficacy established in animals models to treat cataracts, and currently undergoing confirmatory preclinical studies in human lens model to progress to clinical trials (Morton <i>et al.</i> 2013).
PD150606 and PD151746	3-(4-Iodophenyl)-2-mercapto-(<i>Z</i>)-2-propenoic acid, and 3-(5-Fluoro-3-indolyl)-2-mercapto-(<i>Z</i>)-2-propenoic acid	Merck Millipore Co.	Although high selective to calpains in preclinical studies to treat muscular disorders, analogues are under development to favour inhibition of calpain-1 over calpain-2
E-64 and derivatives	[<i>L</i> -trans-3-Carboxyoxirane-2-carbonyl]- <i>L</i> -Leu- <i>agmatine</i>	Sigma Aldrich Co., Merck Millipore Co., Peptide Inst. and Bachem AG.	Safety and efficacy of such compounds had already been demonstrated in preclinical and (Hook <i>et al.</i> 2007) and clinical studies (Satoyashi, 1992), but strategies for enhancing the selectivity for calpain isoforms should be enhanced
Calpain Inhibitor III, MDL-28170 and analogs	Carbobenzoxy-valinyl-phenylalaninal	Merck Millipore Co., Sigma Aldrich Co., Bachem AG. and Enzo Life Sciences Inc.	A powerful and cell-permeable calpain inhibitor (Mehdi, 1991), but presents cross reactivity with cathepsins B and L. Novel highly selective analogues provided promising <i>in vitro</i> results (Kim <i>et al.</i> 2011)
Leupeptin derivatives	Ac-Leu-Leu- <i>L</i> -Argininal	Sigma Aldrich Co., Peptide Inst. and Bachem AG	Derivates of leupeptin are under development as a means of facilitating penetration of the inhibitor into the cells (reviewed by Donkor, 2011).
BDA-410	(2 <i>S</i>)- <i>N</i> -{(1 <i>S</i>)-1-[(<i>S</i>)-Hydroxy(3-oxo-2-phenyl-1-cyclopropen-1-yl)methyl]-2-methylpropyl}-2-benzenesulfonylamino-4-methylpentanamide	Mitsubishi Tanabe Pharma Co.	Relatively selective inhibitor of calpain-1 rather than calpain-2 presenting promising results in Alzheimer's disease in preclinical studies (Battaglia <i>et al.</i> 2003)

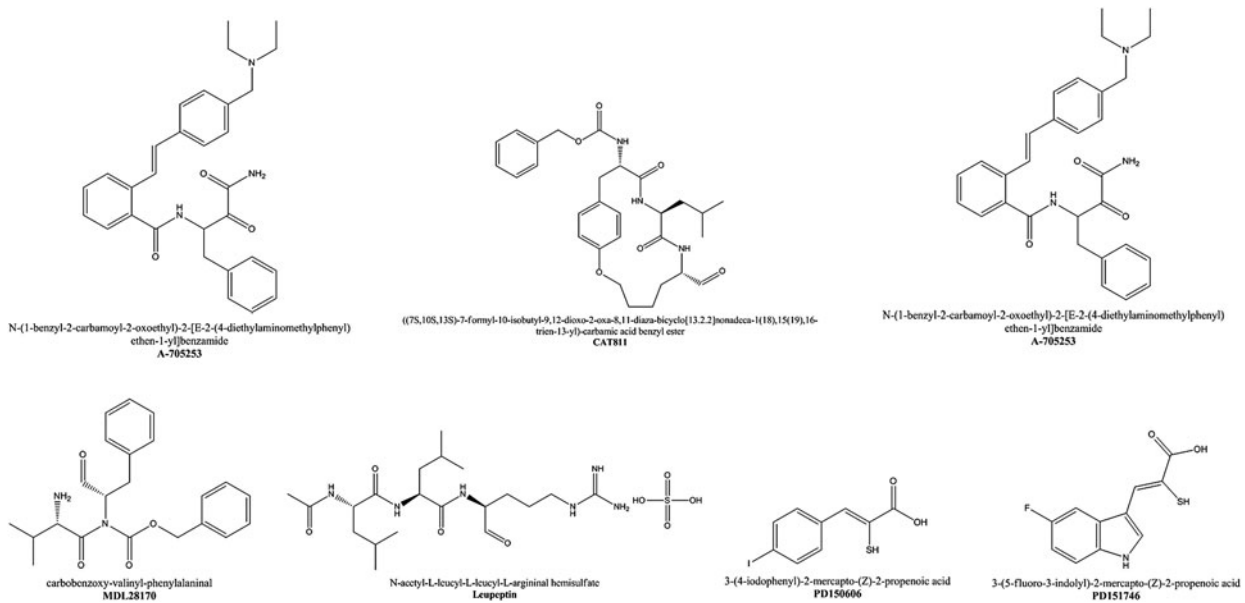


Fig. 2. Chemical structures of selected calpain inhibitors, for an extensive list refer to Donkor (2015).

now, there is no knockout available. However, in *T. brucei*, the RNAi of three calpain genes revealed their roles in parasite growth, morphology and flagellum assembly (Olego-Fernandez *et al.* 2009; Hayes *et al.* 2014).

There are several other evidences on the relevance of calpain molecules for *Leishmania* life cycle disclosed by unbiased assays, such as transcriptomics and proteomics approaches (reviewed by Branquinha *et al.* 2013). When highly sensitive gene expression microarray technology was employed to identify genes that are differentially expressed in *L. donovani* isolated from post kala-azar dermal leishmaniasis (PKDL) patients in comparison with those from visceral leishmaniasis, a 2-fold higher expression of five proteins in PKDL parasites was reported, including a short calpain (Salotra *et al.* 2006). In another approach, a comparative proteomics screen between antimonial-resistant and -sensitive *L. donovani* strains isolated from kala-azar patients revealed a calpain-related protein SKCRP14-1, which is downregulated in the resistant strain, and modulate the susceptibility to antimonials and miltefosine by interfering with drug-induced programmed cell death (PCD) pathways: when over-expressed, this calpain significantly increased the sensitivity of the resistant strain to antimonials, being able to promote PCD, but the opposite effect was seen in miltefosine-treated cells, in which this calpain molecule protected against miltefosine-induced PCD. It was concluded that the calpain SKCRP14-1 is likely to be a regulator of PCD (Vergnes *et al.* 2007). As a matter of fact, conflicting roles for calpain activity in contributing to the promotion and/or suppression of apoptosis have been proposed in mammals, being suggested that calpains must have a wide influence over many apoptotic processes, and their specific roles during apoptosis may differ depending on the cell type and the nature of

the apoptotic stimulus. In *L. major* differentiation from procyclic-into-metacyclic promastigotes, one calpain gene was shown to be upregulated in the procyclic promastigote insect stage, while two distinct calpains were upregulated in the metacyclic insect stage through DNA microarray analysis. Life cycle-specific expression may also demarcate the search for specific functions of these calpains (Saxena *et al.* 2003). In trypanosomatids, it is possible that the great expansion of the calpain family in the parasite genome together with the variety in calpains structure in comparison with the mammalian calpains may contribute to the variety of functions performed (Ersfeld *et al.* 2005; Branquinha *et al.* 2013).

The link between the available calpain inhibitors and a 'me-too' or repurpose approaches for leishmaniasis chemotherapy encouraged our research group to assess the effects of a calpain inhibitor against pathogenic trypanosomatid parasites (d'Avila-Levy *et al.* 2006; Sangenito *et al.* 2009; Ennes-Vidal *et al.* 2010, 2011; Branquinha *et al.* 2013; Marinho *et al.* 2014). MDL28170 (Cbz-Val-Phe-H) is a potent, cell-permeable, synthetic and reversible peptide inhibitor of calpain I and II, also known as calpain inhibitor III (Mehdi 1991). Up to now, this compound showed promising results in pre-clinical studies *in vitro* with some *Leishmania* species. MDL28170 interfered in various steps of the parasite life cycle and incited our research group to program further studies to better understand the calpain functions in these organisms. Our results showed that MDL28170 was capable of arresting irreversibly the growth of *L. amazonensis* promastigotes in a dose-dependent manner (d'Avila-Levy *et al.* 2006), while one possible mechanism of action is through the activation of an apoptotic-like pathway (Marinho *et al.* 2014). Unpublished results from our research group indicate that MDL28170 is also capable of arresting the

development of two *Leishmania* species in host cells. MDL28170 was also effective against all the morphological stages found in *T. cruzi*, including blood-stream trypomastigote, the most infective stage of the parasite (Sangenito *et al.* 2009; Ennes-Vidal *et al.* 2010). In addition, either the treatment of *T. cruzi* with MDL28170 prior to host cell infection, or the post-infection treatment, considerably reduced infection (Ennes-Vidal *et al.* 2010). The calpain inhibitor also arrested the *in vitro* metacyclogenesis of *T. cruzi* and impaired parasite adhesion (epimastigote forms) to the gut of the insect vector *Rhodnius prolixus* in a dose-dependent manner (Ennes-Vidal *et al.* 2011). Interestingly, the compound did not display any relevant cytotoxic effect on mammalian host cells in the concentrations that caused a considerably reduction on the parasite development in the host cells. It is possible that the parasite may concentrate the inhibitor, or even that the diversity of cysteine peptidases in the host cells may surpass the functions blocked by the inhibitor, thus reinforcing the possibilities for chemotherapeutic intervention. Nevertheless, it should be pointed out that MDL28170 as other calpain inhibitors may also act on other parasite peptidases, also the micromolar concentration required for activity may reflect the low affinity to a possible calpain target or even its action on other unpredicted targets. However, its action even in the micromolar ranges encourages the pursuit calpains and calpain inhibitors as a possible chemotherapeutic approach, but with several challenges: identify, purify and characterize an active calpain; determine its structure; identify and select possible inhibitors and test these inhibitors in pre-clinical assays. Also, the new generation of calpain inhibitors should be continuously checked for anti-leishmanial activity.

Concluding remarks

Here, we discuss the repurpose approach as a viable economic alternative to circumvent the bottlenecks of drug discovery pipeline, particularly in neglected disease. There are already examples of a blockbuster success of repurpose drug, such as the Pfizer's Viagra (Sildenafil), which is used to combat erectile dysfunction. Initially, the drug was developed for heart disease treatment, but the observation that penile erections were a common side effect in phase I trials led to pilot studies with Sildenafil in male erectile dysfunction (Ghofrani *et al.* 2006). Interestingly, in *Leishmania*, there is already an example of a repurpose drug. Miltefosine was initially described with antiprotozoal and antineoplastic, however, the application of this compound in an oral formulation in the treatment of different tumours was discontinued, and the successful trials outcomes in India led to approval of the compound for the treatment of visceral leishmaniasis (Dorlo *et al.* 2012). Therefore, a repurpose approach with

approved inhibitors could be an interesting shortcut for the treatment of leishmaniasis, and considering that calpain inhibition is an explored pathway to treat strokes, diabetes and Parkinson's disease, among others, including the possible prevention of spinal cord injury and permanent disability, we advocate that calpain inhibitors should be explored as potential chemotherapeutic agents to treat leishmaniasis. Likewise, other compounds or inhibitors suitable for a repurpose approach should be explored by the scientific community. Also, the calpain family needs more efforts to better characterize it in trypanosomatids, improving not only our knowledge on this intriguing family of peptidases, but also helping in rational drug design approaches.

ACKNOWLEDGEMENTS

The authors are grateful to CAPES, CNPq, FAPERJ and FIOCRUZ fellowships and also to Dr. Malachy McCann from Maynooth University for drawing the chemical structures of the calpain inhibitors.

FINANCIAL SUPPORT

Our research groups are supported by grants from the Brazilian Agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ) to R. S. F. Menna-Barreto, M. H. Branquinha, A. L. S. Santos and C. M. d'Avila-Levy; Fundação Oswaldo Cruz (FIOCRUZ) to R. S. F. Menna-Barreto and C. M. d'Avila-Levy; and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) to C. M. d'Avila-Levy. André L.S. Santos, Marta H. Branquinha, Silvia A. G. da-Silva, Rubem S. F. Menna-Barreto and Claudia M. d'Avila-Levy are also supported by CNPq and FAPERJ fellowships.

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