Antileishmanial activity of MDL 28170, a potent calpain inhibitor

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Abstract

Several calpain inhibitors are under development and some are useful agents against important human pathogens. We therefore investigated the effect of MDL 28170, a potent calpain inhibitor, on the growth of *Leishmania amazonensis*. After 48 h of treatment, the inhibitor exhibited a dose-dependent antileishmanial activity, with a 50% lethal dose (LD_{50}) of 23.3 μM. The inhibitor promoted cellular alterations, such as the parasites becoming short and round. A calpain-like protein migrating at 80 kDa was identified by Western blotting. In addition, the calpain-like molecules were identified on the cell surface of the flagellate. These results add new in vitro insights into the exploitation of calpain inhibitors in treating parasitic infections and add this family of peptidases to the list of potential targets for development of more potent and specific inhibitors against trypanosomatids.

1. Introduction

Leishmaniasis is a group of infectious diseases caused by different species of *Leishmania* that affect about 2 million people per year. Although *Leishmania* is hypoendemic in most of the 88 endemic countries, up to 40% of the population in these countries produces a *Leishmania*-positive skin test in rural endemic areas, illustrating that contact with the parasite is much more widespread than previously thought [1]. The frequency of subclinical cases is well documented for humans and mammalian reservoirs. Indeed, in Brazil the incidence of subclinical cases of leishmaniasis is 10-fold or more higher than the incidence of clinical cases. Infection with human immunodeficiency virus (HIV) can increase the risk of developing leishmaniasis by 100–1000-fold [2]. In addition, visceralisation of *Leishmania* strains such as *Leishmania amazonensis* that are classically restricted to cutaneous leishmaniasis has often been observed in patients with *Leishmania*–HIV co-infection [3]. Clinical reports indicate

that a large proportion of cases are becoming unresponsive to chemotherapy [1,2].

There is a general lack of effective and inexpensive chemotherapeutic agents for treating protozoan diseases that occur mainly in the developing world. Undoubtely one such disease is leishmaniasis. Pentavalent antimonial drugs, such as antimonials, sodium stibogluconate and meglumine antimoniate, are still the first-line treatment for leishmaniasis in most affected areas, with amphotericin B and pentamidine being used as alternative drugs. These agents are expensive, not active orally, require long-term parenteral administration and produce serious side effects (e.g. cardiac and renal toxicity); moreover, resistance to these compounds has become a severe problem. In view of the present clinical scenario, it is desirable that new drugs be developed [4].

Peptidases of microbial pathogens have attracted the attention of many laboratories because of their roles in pathogenesis. Analysis of proteolytic enzymes of pathogenic organisms might lead to the design of powerful chemotherapeutic agents against these pathogens [5,6]. In this context, calpain inhibitors appear an interesting alternative. Calpain is a calcium-regulated cytosolic cysteine peptidase that exists

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in two major isoforms, m-calpain and μ-calpain, which require millimolar and micromolar concentrations of Ca^{2+}, respectively, for their activation [7]. The role of calpain remains poorly understood, but it may be involved in crucial cellular functions such as rearrangement of cytoskeletal proteins and protein cleavage to activate various receptors and pro-enzymes. Calpain activation in humans appears to be increased during normal aging and in muscular dystrophy, cataract, arthritis and Alzheimer's disease, as well as in acute traumas including traumatic brain injury, spinal cord injury and cerebral and cardiac ischaemia. A variety of calpain inhibitors are under development and the potential clinical utility of these inhibitors has been shown in treating Alzheimer's disease [8] and in minimising neuronal death after ischaemia [9]. In addition, these inhibitors are a promising alternative for treating severe acute respiratory syndrome-associated coronavirus (SARSCoV) [10] and adenovirus [11] and may be effective in inhibiting the activation of HIV in latently infected cells [12]. Therefore, these compounds or analogues deserve evaluation as potential therapies for treating leishmaniasis, or could be used as lead compounds for discovery of a more potent *Leishmania* inhibitor. In this context, we have investigated the inhibitory capability of a potent calpain inhibitor, MDL 28170, against *L. amazonensis*, aiming to show its effects on the growth and morphology of the parasite. In addition, we have demonstrated the presence of calpain homologues in *L. amazonensis* by Western blotting, flow cytometry and fluorescence microscopy analyses.

2. Materials and methods

2.1. Chemicals

The calpain inhibitor MDL 28170 (carbobenzoxy-valyl-phenylalanial; Z-Val-Phe-CHO) was purchased from Calbiochem (San Diego, CA). Reagents used in electrophoresis, buffer components, nitrocellulose membranes and reagents for chemiluminescence detection (ECL system) were obtained from Amersham Life Science (Little Chalfont, UK). Dimethyl sulfoxide (DMSO) and Giemsa were acquired from Merck (Darmstadt, Germany). Schneider's medium was obtained from USBiological (Swampscott, MA). Foetal bovine serum (FBS), low molecular mass standard and RPMI medium were purchased from Gibco BRL (Gaithersburg, MD). Thioglycolate, trypan blue and secondary antibodies were purchased from Sigma Chemical Co. (St Louis, MO). All other reagents were of analytical grade.

2.2. Parasite and cultivation

Promastigote forms of *L. amazonensis* Josefa strain (MHOM/BR/75Josefa) were grown in Schneider's medium supplemented with 10% heat-inactivated FBS at 26 °C for 4 days to reach late-log phase growth.

2.3. Effects of the calpain inhibitor MDL 28170 on the growth rate of L. amazonensis

The effects of MDL 28170 on promastigotes of *L. amazonensis* were assessed by a method similar to that described previously [13]. Briefly, promastigotes were counted using a Neubauer chamber and re-suspended in fresh medium to a final concentration of 1.0×10^6 viable promastigotes/mL. Viability was assessed by mobility and lack of staining after challenge with trypan blue. The inhibitor compound was added to the culture at final concentrations of 15, 20, 25 and 30 μM (starting from a 5 mM solution in DMSO that was serially diluted in culture medium). Dilutions of DMSO corresponding to those used to prepare the drug solutions were assessed in parallel. After 24, 48, 72 and 96 h incubation at 26 °C, the number of viable motile promastigotes was quantified daily by counting the flagellates in a Neubauer chamber. Alternatively, parasites grown for 72 h in the absence and presence of the calpain inhibitor were washed five times in cold phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2) prior to re-suspension in drug-free fresh medium and allowed to grow for another 72 h to evaluate the leishmanicidal or leishmanistatic effect. The number of live promastigotes was evaluated as well as cell morphology under optical microscopy at 24-h intervals [14]. The 50% lethal dose (LD_{50}), i.e. the drug concentration that caused a 50% reduction in survival/viability in comparison with that in identical cultures without the compound, was evaluated after 48 h. This value was determined by non-linear regression analysis by plotting the number of viable promastigotes versus log drug concentration using Origin Pro 7.5 computer software.

2.4. Identification of calpain-like molecules by Western blotting

Immunoblot analysis was performed with total cellular extracts from the parasites (200 μg of protein), obtained as previously described [15]. The primary antibodies used were rabbit antisera raised against *Drosophila melanogaster* calpain [16] (anti-Dm-calpain; kindly provided by Dr Yasufumi Emori) and anti-C21, anti-C23 or anti-C24 raised against the whole molecule, the cysteine active site or the histidine active site, respectively, of human brain m-calpain [17] (kindly provided by Dr Ralph Nixon). The secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) at 1:25 000. The membranes were developed by chemiluminescence followed by exposure to radiographic films [15].

2.5. Flow cytometry and immunofluorescence microscopy for calpain detection

Promastigotes (1.0×10^7 cells) used for these experiments were fixed at 4 °C in 0.4% paraformaldehyde in PBS (pH 7.2) for 30 min, followed by extensive washing in the same buffer.

The fixed cells maintained their morphological integrity, as verified by optical microscopy. After this step, the cells were incubated for 1 h at room temperature with a 1:250 or 1:500 dilution of rabbit anti-Dm-calpain polyclonal antibody and then incubated for an additional 1 h with a 1:100 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG [18]. The cells were then washed three times in PBS and observed in a Zeiss epifluorescence microscope (Axioplan 2; Zeiss, Oberkochen, Germany). The images were digitally recorded using a cooled CCD-camera (Color View XS, Analysis GmBH, DE) and analysed with AnalySIS system software. Alternatively, the parasite-associated fluorescence was excited at 488 nm and quantified on a fluorescence-activated cell sorter (FACSCalibur; BD Biosciences, San José, CA) equipped with a 15 mW argon laser emitting at 488 nm. Non-treated cells and those treated with the secondary antibody alone were run in parallel as controls. Each experimental population was then mapped using a two-parameter histogram of forward-angle light scatter versus side scatter. The mapped population ($n = 10\,000$) was then analysed for log green fluorescence using a single-parameter histogram.

2.6. Statistical analysis

All experiments were performed in triplicate in three independent experimental sets. Data were analysed statistically by means of Student's *t*-test using Epi Info 6.04 (Database and Statistics Program for Public Health) computer software. *P*-values ≤ 0.05 were considered statistically significant.

3. Results and discussion

MDL 28170, which is a potent cell-permeable inhibitor of calpain I and II [19], was added to replicating *L. amazonensis* promastigote forms at different concentrations and cell
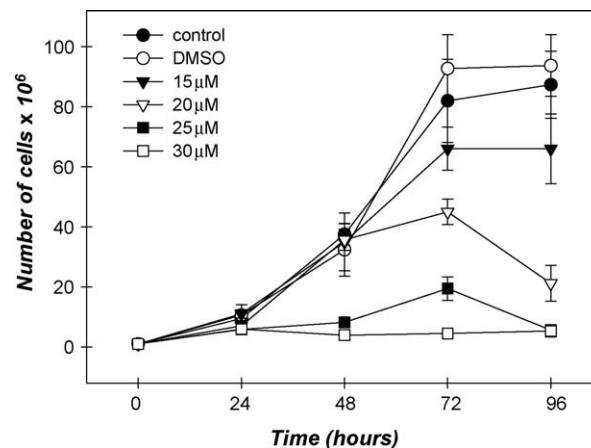

Fig. 1. Effect of MDL 28170, a potent inhibitor of calpain I and II, on the growth rate of *Leishmania amazonensis*. The growth pattern of *L. amazonensis* was followed for parasites cultivated at $26\,^\circ$C in the absence (control) or presence of MDL 28170 at concentrations ranging from 15 μM to 30 μM. The inhibitor was added to the cultures at 0 h and the cells were counted daily. Data shown are the mean ± standard deviation (S.D.) of three independent experiments performed in triplicate. The bars indicate the S.D.

growth was monitored for 4 days in vitro. The results showed that MDL 28170 arrested the growth of *L. amazonensis* in a dose-dependent manner (Fig. 1). The calpain inhibitor at 30 μM induced a powerful reduction in the cellular growth rate by ca. 38%, 90%, 94% and 95% after 24, 48, 72 and 96 h, respectively. The lowest concentrations of the drug (20 μM and 15 μM) presented significant inhibitory effects only after 72–96 h of growth (Fig. 1). Conversely, DMSO did not significantly affect parasite growth behaviour. The LD_{50} after 48 h was 23.3 ± 0.9 μM.

The antileishmanial activity was irreversible, since protozoa pre-treated for 72 h with the calpain inhibitor at 30 μM did not resume growth when subcultured in fresh medium (data not shown). Corroborating these results, opti-
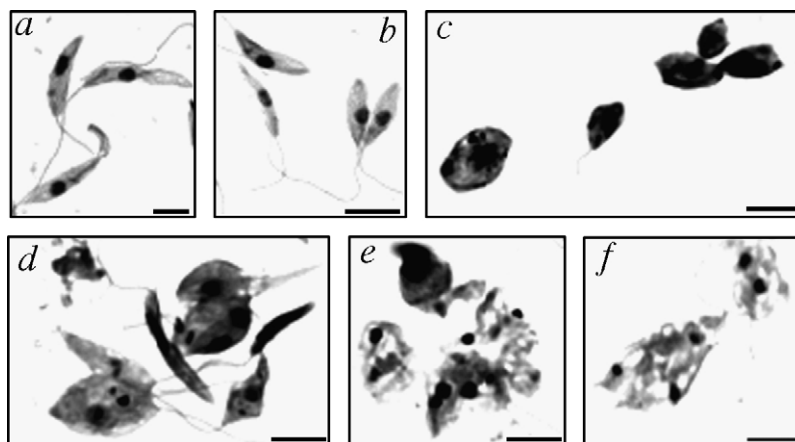

Fig. 2. Microscopic observations of the viability of promastigote forms of *Leishmania amazonensis* incubated in the absence or presence of MDL 28170. (a, b) Control cells cultured in Schneider's medium (a) or in the presence of dimethyl sulfoxide at a dose used to dissolve the highest concentration of MDL 28170 (b). (c–f) Cells cultured in the presence of 15 μM (c), 20 μM (d), 25 μM (e) or 30 μM (f) of MDL 28170. Note the increase in cell volume (c, d) and complete lysis of the parasite cells (e, f). The bar represents 1 μM.
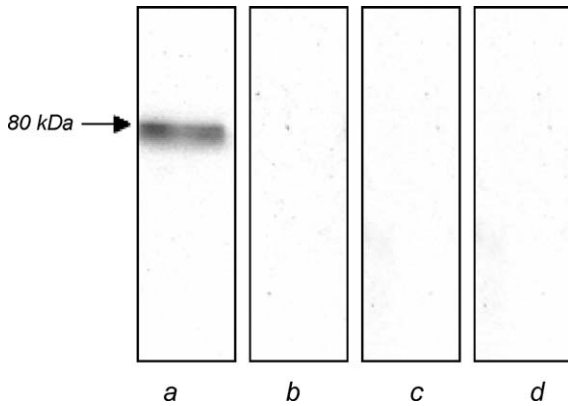
Fig. 3. Western blotting analysis of polypeptides from *Leishmania amazonensis* probed with anti-Dm-calpain (lane a), anti-C21 (lane b), anti-C23 (lane c) and anti-C24 (lane d). The number on the left indicates the apparent molecular mass of the reactive polypeptide.
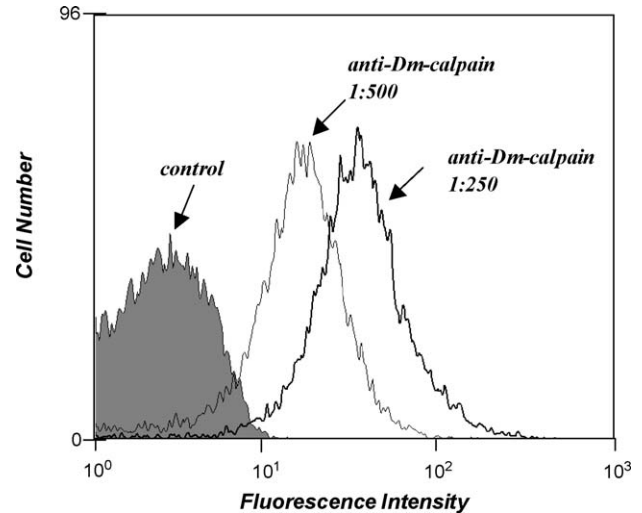

Fig. 4. Flow cytometric analysis showing the anti-Dm-calpain antibody binding to *Leishmania amazonensis*. Paraformaldehyde-fixed cells were incubated in the absence (control) or presence of anti-Dm-calpain antibody at 1:250 or 1:500 dilutions, as described in Section 2.5, and then analysed by flow cytometry. Representative data from analysis of 10 000 cells from one of three experiments are shown.

cal microscopy observations showed a massive deterioration of promastigote cells after treatment of the parasites with 30 μM MDL 28170 for 48 h (Fig. 2f) compared with the untreated (Fig. 2a) and DMSO-treated cells (Fig. 2b). In addition, treatment with lower concentrations of the calpain inhibitor initially promoted an increase in the cell volume (Fig. 2c and d) and then lysis of the parasite cell (Fig. 2e).

Based on the effects of MDL 28170 on the growth rate and morphology of *L. amazonensis*, we aimed to detect calpain homologues in this protozoan by immunoblot assays using different anti-calpain antibodies (Fig. 3). Anti-Dm-calpain strongly recognised a polypeptide band migrating at approximately 80 kDa (Fig. 3, lane a). No common epitopes were found between mammalian calpains and *L. amazonensis* polypeptides (Fig. 3, lanes b–d). Lysates of mouse liver were recognised by anti-C21, anti-C23,

anti-C24 and anti-Dm-calpain antibodies (data not shown). The calpain-like molecule was detected on the cell surface of *L. amazonensis*, as demonstrated by flow cytometry and fluorescence microscopy analyses using the anti-Dm-calpain antibody (Figs 4 and 5). The immunofluorescence image showed labelling throughout the cell surface, including the flagellum. These results suggest that this trypanosomatid possesses molecules that share antigens with invertebrate calpain-related enzymes.

In trypanosomatids, a calpain-related protein has already been reported in *Trypanosoma brucei* [20] and *Crithidia deanei* [21]. In the former, the protein was shown to be
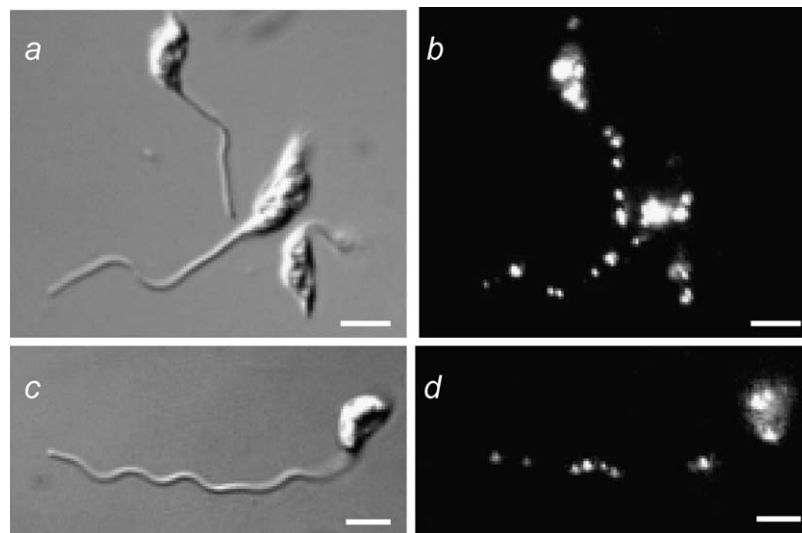

Fig. 5. Fluorescence microscopy showing labelling of *Leishmania amazonensis* with anti-Dm-calpain antibodies. Experimental systems were analysed under differential interferential contrast images (a, c) and immunofluorescence (b, d). The bar represents 1 μm.

expressed in procyclics but not in bloodstream trypomastigotes. Furthermore, the protein probably possesses a stable interaction with the cell membrane, suggesting a function in cellular remodelling [20]. In *C. deanei*, the calpain-related molecule was shown to be proteolytically active and to be released by cells, however its function was not determined [21]. In this context, the inhibition of leishmanial growth produced by MDL 28170 may be due to its effects on calpain-like molecules. These results add new in vitro insights into the exploitation of calpain inhibitors in treating parasitic infections and add this family of proteases to the list of potential targets for development of more potent and specific inhibitors.

Acknowledgments

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