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Short Communication

HIV aspartic peptidase inhibitors are effective drugs against the trypomastigote form of the human pathogen *Trypanosoma cruzi*

Leandro S. Sangenito [a], Diego S. Gonçalves [a,b], Sergio H. Seabra [c], Claudia M. d'Avila-Levy [d], André L.S. Santos [a,b,*,1], Marta H. Branquinha [a,*,1]

[a] *Laboratório de Investigação de Peptidases, Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes (IMPG), Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil*
[b] *Programa de Pós-Graduação em Bioquímica, Instituto de Química, UFRJ, Rio de Janeiro, Brazil*
[c] *Laboratório de Tecnologia em Cultura de Células, Centro Universitário Estadual da Zona Oeste (UEZO), Rio de Janeiro, Brazil*
[d] *Laboratório de Estudos Integrados em Protozoologia, Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil*

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ABSTRACT

There is a general lack of effective and non-toxic chemotherapeutic agents against Chagas' disease despite more than a century of research. In this regard, we have verified the impact of human immunodeficiency virus aspartic peptidase inhibitors (HIV-PIs) on the viability and morphology of infective trypomastigote forms of *Trypanosoma cruzi* as well as on the aspartic peptidase and proteasome activities produced by this parasite. The effects of HIV-PIs on viability were assessed by counting motile parasites in a Neubauer chamber. Morphological alterations were detected by light microscopy of Giemsa-stained smears and scanning electron microscopy. Modulation of aspartic peptidase and proteasome activities by the HIV-PIs was measured by cleavage of fluorogenic peptide substrates. The majority of the HIV-PIs (6/9) were able to drastically decrease the viability of trypomastigotes after 4 h of treatment, with nelfinavir and lopinavir being the most effective compounds presenting LD_{50} values of 8.6 μM and 10.6 μM, respectively. Additionally, both HIV-PIs were demonstrated to be effective in a time- and cell density-dependent manner. Treatment with nelfinavir and lopinavir caused many morphological/ultrastructural alterations in trypomastigotes; parasites became round in shape, with reduced cell size and flagellar shortening. Nelfinavir and lopinavir were also capable of significantly inhibiting the aspartic peptidase and proteasome activities measured in trypomastigote extracts. These results strengthen the data on the positive effects of HIV-PIs on parasitic infections, possibly by targeting the parasite aspartic peptidase(s) and proteasome(s), opening a new possibility for the use of these clinically approved drugs as an alternative chemotherapy to treat Chagas' disease.

1. Introduction

Trypanosoma cruzi, the causative agent of Chagas' disease, is responsible for more morbidity and mortality in Latin America countries than any other parasitic disease [1]. Moreover, 20–30% of infected individuals will develop the chronic form of the disease, which includes Chagas' heart disease as well as gastrointestinal and neuronal disorders [1]. *T. cruzi* is predominantly transmitted by triatomine insects; however, the international migration process and diverse parasite transmission possibilities, such as blood transfusion, have enabled the spread of infected individuals around the world [1,2]. Furthermore, co-infection with *T. cruzi* and human immunodeficiency virus (HIV) leads to reactivation of the parasitic infection, with exacerbation of clinical signs and unusual manifestations [2].

Clinical treatment of Chagas' disease is a huge issue to be solved, since only two drugs are currently approved, namely benznidazole and nifurtimox, which cause severe side effects that can lead to premature termination of therapy [1]. Consequently, new strategies must be explored to combat this neglected illness. Interestingly, some promising and beneficial properties of aspartic peptidase inhibitors (PIs) used in the current chemotherapy against acquired immune deficiency syndrome (AIDS) have been described effecting crucial biological events of opportunistic protozoan parasites that cause considerably morbimortality in HIV patients [3–5]. Regarding *T. cruzi*, a preliminary study by our group showed that the classic aspartic PI pepstatin A effectively reduced the proliferation of epimastigotes

* Corresponding authors. Present address: Laboratório de Investigação de Peptidases (LIP), Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes (IMPG), Centro de Ciências da Saúde (CCS), Universidade Federal do Rio de Janeiro (UFRJ), Bloco E-subsolo, sala 05, Rio de Janeiro, RJ 21941-902, Brazil. Fax: +55 21 3938 8344.
E-mail addresses: andre@micro.ufrj.br (A.L.S. Santos); mbranquinha@micro.ufrj.br (M.H. Branquinha).
[1] These two authors share senior authorship.

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(insect stage) [6]. In a subsequent report, the use of HIV-PIs arrested epimastigote growth and caused many ultrastructural alterations [5]. In addition, the interaction process of epimastigotes with *Rhodnius prolixus* midgut was blocked by HIV-PIs [5]. Owing to these results, in the present paper the effect of nine clinically approved HIV-PIs on the infective trypomastigotes of *T. cruzi* as well as on the hydrolytic activity of aspartic peptidases and proteasomes produced by this parasite was evaluated.

2. Materials and methods

2.1. Parasite and cultivation

Trypomastigotes of *T. cruzi* Y strain were harvested from culture supernatants of infected LLC-MK_2 epithelial cells after 5 days of incubation in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St Louis, MO) supplemented with 2% foetal bovine serum (FBS) (Gibco; Life Technologies, Grand Island, NY) at 37 °C in 5% CO_2.

2.2. Effects of aspartic peptidase inhibitors on trypomastigote viability and morphology

Trypomastigotes (10^6 parasites/mL) were incubated in DMEM containing 2% FBS at 37 °C in the absence or presence of aspartic PIs (pepstatin A and nine HIV-PIs) (Supplementary Fig. S1) and benznidazole at final concentrations ranging from 1 μM to 50 μM. Parasite viability was monitored every hour until 4 h by motility and lack of Trypan blue staining. The 50% lethal dose (LD_{50}) was determined after 4 h by linear regression. Subsequently, the effects of the two most efficacious aspartic PIs were assessed on the viability rate at different parasite densities (10^5–10^7 viable cells/mL). Light microscopy examination was also performed following Giemsa staining.

2.3. Effect of HIV aspartic peptidase inhibitors on ultrastructure

Trypomastigotes were incubated in DMEM with 2% FBS in the absence or presence of nelfinavir or lopinavir at LD_{50} values for 4 h. For scanning electron microscopy (SEM) observation, parasites were processed as described previously and were observed in a JEOL JSM 6490LV scanning electron microscope (JEOL USA Inc., Peabody, MA) [7].

2.4. Effect of HIV aspartic peptidase inhibitors on aspartic peptidase activity

The enzymatic activity over cathepsin D substrate [7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg amide] was determined using trypomastigote extracts (40 μg of protein) in the presence or absence of pepstatin A, nelfinavir and lopinavir at 10 μM [5]. Cleavage of cathepsin D substrate was monitored continuously at 37 °C for 1 h in a spectrofluorometer (SpectraMax Gemini XPS; Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 328 nm and an emission wavelength of 393 nm.

2.5. Effect of HIV aspartic peptidase inhibitors on proteasome activity

The enzymatic activity over the proteasome substrate (carbobenzoxy-Gly-Gly-Leu-7-amide-4-methylcoumarin) was determined using trypomastigote extracts, which were obtained by freeze–thawing cycles of 10^8 cells in a buffer containing 25 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM ethylene diamine tetra-acetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM tosyl phenylalanyl chloromethyl ketone (TPCK), 1 mM tosyl-L-lysine chloromethyl ketone (TLCK)and 20 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) (all from Sigma-Aldrich). The assay was performed by mixing the parasite extract (50 μg of protein) with the proteasome substrate (13 μM) in reaction buffer [50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT and 1 mM ATP]. The samples were incubated at 37 °C for 1 h in the presence or absence of 50 μM nelfinavir, lopinavir or the proteasome inhibitor Z-LLF-CHO. Chymotrypsin-like activity was determined at an excitation wavelength of 380 nm and an emission wavelength of 440 nm in a SpectraMax Gemini XPS spectrofluorometer.

2.6. Statistical analysis

All experiments were performed in triplicate in three independent experimental sets. Data were analysed statistically by means of one-way analysis of variance (ANOVA) using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

3. Results and discussion

Most of the aspartic PIs (6/10; 60%) were very effective in reducing the viability of trypomastigotes under the employed conditions (Fig. 1A). Atazanavir, amprenavir, tipranavir and pepstatin A were not active against trypomastigotes in the concentration range employed. The LD_{50} values for saquinavir, ritonavir, indinavir and darunavir were calculated as 12.3, 18.6, 31.7 and 47.4 μM, respectively. Nelfinavir and lopinavir were the most effective aspartic PIs tested, presenting LD_{50} values of 8.6 μM and 10.6 μM, respectively (Fig. 1A). These latter two HIV-PIs presented a similar efficacy compared with the classical drug benznidazole, for which the LD_{50} was 6.9 μM (data not show). Also, nelfinavir and lopinavir reduced the viability of trypomastigotes in a typical time-, dose- and parasite density-dependent manner (Fig. 1B,C). Comparatively, nelfinavir and lopinavir were also capable of suppressing the viability of *T. cruzi* epimastigotes after 4 h, presenting LD_{50} values of 31.9 μM and 29.1 μM, respectively (data not shown), when cultivated under the same conditions employed for trypomastigotes. As a matter of fact, these data clearly indicate that the infective form is more susceptible to HIV-PIs than the insect stage, which encouraged the forthcoming studies. In addition, the results are in accordance with the previous observation of the effectiveness of HIV-PIs in suppressing the in vitro proliferation of several species of *Leishmania* that cause both cutaneous and visceral leishmaniasis [3,8–10].

A simple inspection by means of light microscopy analysis corroborated the anti-*T. cruzi* action of HIV-PIs, in which the treatment of trypomastigotes with nelfinavir and lopinavir revealed some relevant morphological changes compared with non-treated cells (Fig. 2A). The most significant alterations were rounding in shape with reduced cell size, swollen cell body, and shortening and/or loss of flagellum. As the concentration of each drug increased, these morphological changes became more frequent and drastic (Fig. 2A). These cellular alterations were comparable with those caused by pepstatin A [6] and HIV-PIs [5] in *T. cruzi* epimastigotes. Diazoacetylnorleucine methyl ester (DAN), another aspartic PI, caused progressive modifications on the shape of *Leishmania mexicana* promastigotes, from a long slender form to a spherical one with two or three nuclei/cell [11].

SEM of the HIV-PI-treated trypomastigotes was performed to further analyse the ultrastructural changes (Fig. 2B). Parasites treated with the LD_{50} values of nelfinavir and lopinavir showed different degrees of morphological changes compared with control cells (Fig. 2B,a). Many parasites exhibited shrinkage and cell rounding as well as shortening of the flagellum as the main responses to the treatment, alterations that were consistent with the decrease in their viability (Fig. 2B,c,e). Moreover, some parasites presented membrane protrusions resembling surface blebs and ruffling of the plasma