

Nelfinavir is effective in inhibiting the multiplication and aspartic peptidase activity of *Leishmania* species, including strains obtained from HIV-positive patients

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Objectives: There is a general lack of effective and non-toxic chemotherapeutic agents for leishmaniasis and there is as yet no study about the effect of HIV peptidase inhibitors (HIV PIs) on *Leishmania*/HIV-coinfected patients. In the present work, we performed a comparative analysis of the spectrum of action of HIV PIs on different *Leishmania* spp., including strains obtained from HIV-positive patients receiving or not receiving antiretroviral treatment.

Methods: The effects of nelfinavir and saquinavir on *Leishmania* proliferation were assessed by means of a colorimetric assay (MTT). Subsequently, the effect of nelfinavir on aspartic peptidase activity from *Leishmania* spp. was assessed by following the degradation of the fluorogenic substrate MCA-G-K-P-I-L-F-F-R-L-K-DNP-Arg-NH₂.

Results: Nelfinavir was capable of significantly reducing the multiplication of many *Leishmania* reference strains and isolates obtained from HIV-positive patients receiving or not receiving antiretroviral treatment. *Leishmania major* growth was inhibited by ~50%, while all other flagellates were strongly inhibited (at least 94%), except for a *Leishmania chagasi* strain obtained from an HIV-positive patient under treatment with highly active anti-retroviral therapy (HAART). Culture of this isolate in the presence of nelfinavir induced a considerable reduction in the aspartic peptidase activity. In addition, nelfinavir was also capable of inhibiting the aspartic peptidase activity of all *Leishmania* strains tested.

Conclusions: The present data contribute to the study of the effect of HIV PIs on *Leishmania* infection and add new insights into the possibility of exploiting aspartic peptidases as promising targets in order to generate novel medications to treat leishmaniasis.

Keywords: aspartyl peptidases, *Leishmania*/HIV coinfection, HIV peptidase inhibitors, chemotherapy, leishmaniasis, proteases

Introduction

The treatment of leishmaniasis is still based on an empirical treatment with pentavalent antimonials developed more than 50 years ago. In this treatment, poor therapeutic responses and adverse effects are common. Nowadays, leishmaniasis is also considered an opportunistic disease in patients infected with HIV-1. In these patients, treatment with highly active anti-retroviral therapy (HAART) has shown a strong reduction in opportunistic infections, including those caused by parasites.¹

However, patients coinfecting with visceral leishmaniasis and HIV could be a reservoir for the development and spread of drug-resistant strains.² In addition, it is clear that patients that do not receive HAART are more likely to develop the pathology of leishmaniasis and show an increased risk of failure in AIDS treatment.^{3–7} HAART uses multiple anti-HIV drugs, including aspartic peptidase inhibitors (PIs). The aspartic peptidases are not well characterized in the Trypanosomatidae family. Soluble extracts of *Leishmania amazonensis* are capable of degrading synthetic substrates designed for aspartic peptidases; such

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degradation is abolished by selective aspartic peptidase inhibitors.^{8–11} In our recent article, we showed that nelfinavir and lopinavir, two HIV PIs that are aspartic peptidase inhibitors, cause major changes in several crucial steps of the life cycle of *L. amazonensis*, such as proliferation, invasion of macrophages, ultrastructural aberrations and expression of virulence factors.¹⁰ The effect of HIV PIs on *Leishmania* proliferation was also observed on other species of *Leishmania*, including *Leishmania infantum*, *Leishmania donovani*, *Leishmania major*, *Leishmania mexicana* and *Leishmania braziliensis*.^{11–13} There is a general lack of consensus about the susceptibility of distinct *Leishmania* species to the HIV PIs. It is as yet unclear if the discrepancies in previous reports are due to differences in the methodologies used, strains and species assayed, or drug origin. Therefore, our main aim was to perform a comparative analysis of the spectrum of action of the HIV PIs nelfinavir and saquinavir on different *Leishmania* spp., including strains obtained from HIV-positive patients receiving or not receiving antiretroviral treatment. We also assessed aspartic peptidase activity among these isolates and its susceptibility to HIV PIs, and tested the influence of nelfinavir on aspartic peptidase activity of *Leishmania chagasi* through successive passages *in vitro*.

Materials and methods

Chemicals

The HIV PIs saquinavir and nelfinavir were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. DMSO, heat-inactivated fetal bovine serum (FBS), MTT, dithiothreitol (DTT), EDTA, Schneider's insect medium, 3-[(3-cholamidopropyl)-dimethylammonio]-1 propanesulphonate (CHAPS) and cathepsin D fluorogenic substrate [7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-amide; hereafter referred to as MCA] were purchased from Sigma Chemicals. All other reagents were of analytical grade or superior.

Parasite culture

L. amazonensis (MHOM/BR/77/LTB0016), *L. braziliensis* (MCAN/BR/1998/619), *L. chagasi* (MHOM/BR/1974/PP75), *L. donovani* (MHOM/ET/1967/L82;HV3;LV9), *L. major* (MHOM/IL/1980/FRIEDLIN), *L. chagasi* obtained from an untreated HIV-positive patient (MHOM/BR/2009/ANC), *L. chagasi* obtained from an HIV-positive patient under antiretroviral treatment #1 (MHOM/BR/2009/LCS) and *L. chagasi* obtained from an HIV-positive patient under antiretroviral treatment #2 (MHOM/BR/2009/VCF) (Table 1) were obtained from the *Leishmania* Type Culture Collection (Fundação

Oswaldo Cruz, Rio de Janeiro, RJ, Brazil). Promastigote forms were maintained by weekly transfers in 25 cm² culture flasks with Schneider's insect medium, pH 7.0, supplemented with 10% FBS at 28°C. In the drug-induced pressure experiment, the parasites were subjected to 10 successive *in vitro* passages in medium supplemented with 50 µM nelfinavir.

Multiplication inhibition assay

The effects of two distinct HIV PIs, nelfinavir and saquinavir, on the viability of promastigote forms of several *Leishmania* isolates were assessed by means of the MTT assay. Promastigotes obtained from a log-phase culture (1.0×10⁷ cells) were resuspended in fresh medium (200 µL) supplemented or not with 25 µM saquinavir or nelfinavir. A dilution of DMSO corresponding to that used to prepare the drug solutions was assessed in parallel. After 72 h of incubation at 28°C, the number of viable promastigotes was quantified by addition of MTT solution (5 mg/mL in PBS, 50 µg/well), and the plates were then incubated for 3 h in the dark at 37°C. The plates were subsequently centrifuged at 672 g for 7 min, the supernatant was removed, the pellet was dissolved in 200 µL of DMSO and absorbance was measured in an ELISA reader at 490 nm (Bio-Tek Instruments).¹⁴

Aspartic peptidase assay

The enzymatic activity on the cathepsin D substrate was determined using parasite extracts obtained by repeated freeze–thawing cycles of cells in 10 mM Tris–HCl, pH 7.2, containing 1% CHAPS. Then, the cellular extract was incubated for 40 min at 4°C, centrifuged (10000 g for 30 min at 4°C) and stored at –70°C in aliquots for no longer than 5 days. Cleavage of cathepsin D substrate was monitored continuously in a spectrofluorimeter (SpectraMax Gemini XPS, Molecular Devices, CA, USA) using an excitation wavelength of 328 nm and an emission wavelength of 393 nm. A 12 µM stock solution of the fluorogenic substrate sample was prepared in DMSO. The reaction was started by the addition of 2 µM substrate to the parasite extract (10 µg) in a total volume of 60 µL of 100 mM sodium acetate, 1 M sodium chloride, 1 mM EDTA, 1 mM DTT, 10% DMSO, 1 mg/mL BSA, pH 4.7, in the presence or absence of 1 or 10 µM nelfinavir or saquinavir. The reaction mixture was incubated at 37°C for 30 min. The assays were controlled for self-liberation of the fluorophore over the same time interval.¹⁰

Statistical analysis

All experiments were carried out at least three times in triplicate. Data on the effect of HIV PIs on *Leishmania* species were analysed statistically by Student's *t*-test using EPI-INFO computer software. *P* values of ≤0.05 were considered statistically significant.

Table 1. Drugs used in the treatment of HIV-positive patients

Drug	Drug class	Treatment #1	Treatment #2
Zidovudine	nucleoside reverse transcriptase inhibitors	yes	
Lamivudine	nucleoside reverse transcriptase inhibitors	yes	yes
Enfuvirtide	entry and fusion inhibitors		yes
Tenofovir	nucleoside reverse transcriptase inhibitors		yes
Darunavir	peptidase inhibitors		yes
Kaletra® (combination of lopinavir and ritonavir)	peptidase inhibitors		yes

Ethics approval

The protocols described used in this study were approved by the Ethics Committee of Hospital Universitário Federal do Maranhão (No. 003283/2009-90) according to the Declaration of Helsinki of 1975, revised in 1983, and individuals who agreed to participate signed a proper informed consent form.

Results

Multiplication inhibition assay

We tested the effect of HIV PIs on the growth of promastigotes in order to establish the differences in susceptibility among several *Leishmania* species, as well as strains obtained from HIV-positive patients under distinct treatments (Table 2). Nelfinavir and saquinavir were added to replicating promastigote forms of *Leishmania* species at a final concentration of 25 μ M and cellular growth was determined by the MTT assay after 72 h of incubation. The HIV PI nelfinavir was able to significantly inhibit the growth of all strains tested by at least 94%, with two exceptions: *L. chagasi* obtained from an HIV-positive patient under antiretroviral treatment #2, which showed no reduction, and *L. major*, which presented an intermediate inhibitory level of ~50% (Table 2). When we used the HIV PI saquinavir, only *L. donovani* growth was significantly altered, corresponding to 62.2% of inhibition in relation to the control. A comparison of these data with previous studies on *Leishmania* inhibition by HIV PIs is shown in Table 3.

Effect of HIV PIs on *Leishmania* aspartic peptidase

Synthetic peptide substrates can be used as an effective tool for the identification and biochemical characterization of different peptidases in microorganisms.^{15,16} In this work, a cathepsin D substrate was used to assess the aspartic peptidase activity in extracts of several *Leishmania* species. Additionally, the inhibitory capability of the HIV PIs nelfinavir and saquinavir was tested at 1 or 10 μ M. All *Leishmania* spp. extracts were able to degrade the cathepsin D substrate at pH 4.7, and showed dose-dependent inhibition by nelfinavir (Figure 1); however, saquinavir presented no

significant inhibition of the degradation rate of the substrate (data not shown). A comparison of the degradation rate among the assayed *L. chagasi* strains reveals interesting differences. The strain isolated from a patient under treatment with HIV PIs presented the lowest rate of substrate hydrolysis. This finding motivated us to investigate whether nelfinavir would be able to negatively modulate the activity of aspartic peptidase in *Leishmania*. We therefore performed 10 successive *in vitro* passages of *L. chagasi* in the presence of nelfinavir, and observed that this strain showed a significant decrease, of ~36.2%, in activity against the aspartic peptidase substrate (Figure 2).

Discussion

The introduction of HAART has dramatically changed the course of HIV infection. In AIDS patients, the incidence of coinfections caused by bacteria, fungi and protozoa and the morbidity and mortality due to these coinfections have declined significantly.^{1,11} Although in most cases these improvements have been attributed to HAART-induced recovery of host immunity, improvements in opportunistic infections have been demonstrated even in the absence of immunological recovery.¹⁷⁻²⁰ There is evidence that the HIV PIs included in HAART not only restore cell-mediated immunity but also have a direct inhibitory effect on the peptidases of certain pathogens.¹ The influence of HIV PIs on *Leishmania* multiplication has been tested previously using the compounds nelfinavir and saquinavir. However, there seems to be a complete lack of consensus on the susceptibilities of different *Leishmania* species, strains and isolates to the available HIV PIs (Table 3). These discrepancies could be the consequence of discrete differences between *Leishmania* isolates²¹ and differences in methodology, drug origin and so on. Therefore, the present report assessed a panel of distinct *Leishmania* spp. and strains under the same conditions.

L. chagasi and *L. infantum* are the aetiological agents of visceral leishmaniasis and there is strong evidence for a close similarity between *L. infantum* and *L. chagasi*,²²⁻²⁶ and more recently this hypothesis was confirmed by multilocus microsatellite typing.²⁷ In this context, we showed here that *L. chagasi* could be significantly inhibited by nelfinavir but not by saquinavir at 25 μ M

Table 2. Effect of nelfinavir and saquinavir on *in vitro* proliferation of *Leishmania* promastigotes

Source	Species	Percentage of growth inhibition ^a	
		nelfinavir	saquinavir
Reference strains	<i>L. amazonensis</i> (MHOM/BR/77/LTB0016)	95.5*	5.4
	<i>L. braziliensis</i> (MCAN/BR/1998/619)	95.6*	13.6
	<i>L. donovani</i> (MHOM/ET/1967/L82;HV3;LV9)	94*	62.2*
	<i>L. major</i> (MHOM/IL/1980/FRIEDLIN)	50*	21.2
	<i>L. chagasi</i> (MHOM/BR/1974/PP75)	96.2*	0
HIV-positive patient strains	<i>L. chagasi</i> /no treatment (MHOM/BR/2009/ANC)	96.6*	0.4
	<i>L. chagasi</i> /treatment #1 (MHOM/BR/2009/LCS)	96.3*	0
	<i>L. chagasi</i> /treatment #2 (MHOM/BR/2009/VCF)	0	0

^aCells were treated with 25 μ M saquinavir or nelfinavir for 72 h at 28°C, and the number of viable promastigotes was quantified by means of the MTT colorimetric assay. Values are percentages of inhibition relative to respective controls. Asterisks denote systems treated with HIV PIs that showed inhibition significantly different from the control ($P \leq 0.05$; Student's *t*-test). For details of treatment #1 and #2 see Table 1.

Table 3. Comparative table of *Leishmania* growth inhibition by the HIV PIs saquinavir and nelfinavir reported here and in previous studies

Species/isolates	Saquinavir		Nelfinavir		Reference
	concentration (μM)	growth inhibition	concentration (μM)	growth inhibition	
<i>L. amazonensis</i> (MHOM/BR/77/LTB016)	25	5.4%	25	95%	this report
<i>L. amazonensis</i> (MHOM/BR/77/LTB016)	50	NSI	15.12	50%	10
<i>L. amazonensis</i> (IFLA/BR/67/PH8)	40	50%	13.36	50%	11
<i>L. major</i> (MHOM/IL/1980/FRIEDLIN)	25	21.2%	25	50%	this report
<i>L. major</i> (LRC-L137)	7	50%	NA	NA	12
<i>L. major</i> (MHOM/SU/73/5-ASKH)	46.95	50%	13.37	50%	11
<i>L. chagasi</i> (MHOM/BR/1974/PP75)	25	NSI	25	96.2%	this report
<i>L. chagasi</i> (MHOM/BR/2009/ANC) ^a	25	NSI	25	96.6%	this report
<i>L. chagasi</i> (MHOM/BR/2009/LCS) ^a	25	NSI	25	96.3%	this report
<i>L. chagasi</i> (MHOM/BR/2009/VCF) ^a	25	NSI	25	NSI	this report
<i>L. infantum</i> (MHOM/MA/67/ITMAP-253)	25	NSI	25	NSI	13
<i>L. infantum</i> (MHOM/TN/80/IPT1)	50	31%	NA	NA	12
<i>L. infantum</i> (MHOM/FR/78/LEM-75)	53.97	50%	16.46	50%	11
<i>L. infantum</i> (MCAN/ES/98/LLM-724)	50.87	50%	17.59	50%	11
<i>L. infantum</i> (MCAN/VE/98/IBO-78)	55.12	50%	14.05	50%	11
<i>L. infantum</i> (MHOM/ES/95/LLM-480)	48.04	50%	18.21	50%	11
<i>L. infantum</i> (MHOM/ES/98/LLM-759) ^a	64.46	50%	26.89	50%	11
<i>L. braziliensis</i> (MCAN/BR/1998/619)	25	13%	25	95%	this report
<i>L. braziliensis</i> (MHOM/BR/75/M2903)	36	50%	14.6	50%	11
<i>L. donovani</i> (MHOM/ET/1967/L82;HV3;LV9)	25	62.2%	25	94%	this report
<i>L. donovani</i> (MHOM/IN/80/DD8)	51.89	50%	14.1	50%	11

NSI, no significant inhibition; NA, not assessed.

^aStrains isolated from HIV/*Leishmania*-coinfected patients.

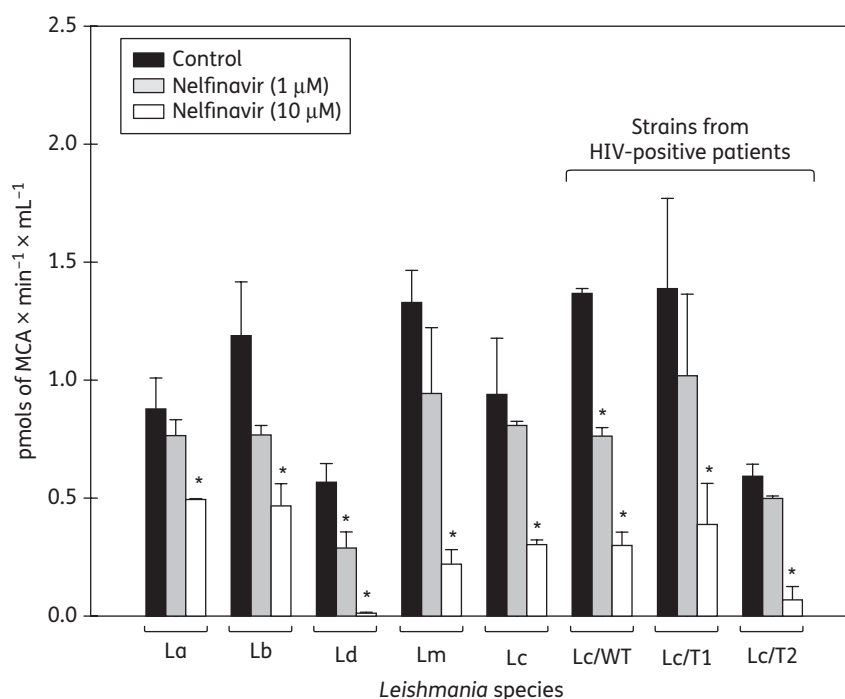


Figure 1. Effect of the HIV PI nelfinavir on *Leishmania* aspartic peptidase activity. Proteolytic activity was assessed using a cathepsin D fluorogenic substrate (MCA). Inhibition of proteolytic activity was tested with nelfinavir at 1 or 10 μM . La, *L. amazonensis*; Lb, *L. braziliensis*; Ld, *L. donovani*; Lm, *L. major*; Lc, *L. chagasi*; Lc/WT, *L. chagasi* obtained from an HIV-positive patient without treatment; Lc/T1, *L. chagasi* obtained from an HIV-positive patient under treatment #1; Lc/T2, *L. chagasi* obtained from an HIV-positive patient under treatment #2 (see Table 1). Bars indicate standard errors of the mean. Asterisks indicate P values ≤ 0.05 .

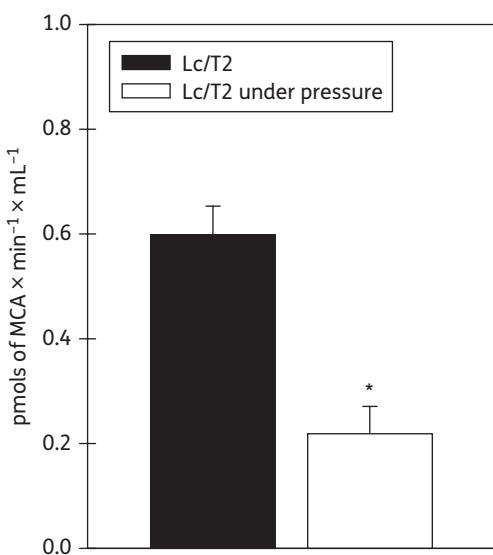


Figure 2. Decrease in the degradation rate of an aspartic peptidase substrate by an *L. chagasi* strain subjected to nelfinavir *in vitro* pressure. Proteolytic activity was assessed through a cathepsin D fluorogenic substrate (MCA) and compared between freshly isolated parasites (Lc/T2) and parasites subjected to 10 successive passages in culture medium supplemented with nelfinavir (Lc/T2 under pressure). Lc/T2, *L. chagasi* isolated from an HIV-positive patient under treatment #2 (see Table 1). Bars indicate standard errors of the mean. The asterisk indicates a P value ≤ 0.05 .

(Table 2). Accordingly, Trudel et al.¹³ reported that saquinavir was not able to inhibit the multiplication of *L. infantum* promastigotes, but, in contrast to their observations, we found that nelfinavir at 25 μ M caused a strong reduction of 96.6% in *L. chagasi* growth. Corroborating this finding, Savoia et al.¹² reported inhibition by nelfinavir of *L. infantum* growth ranging from 5% to 34% in a dose-dependent manner.¹¹ In spite of the controversy in the literature about *Leishmania* susceptibility to HIV PIs (Table 3), there seems to be a consensus about saquinavir's lower effectiveness in comparison with nelfinavir. Here, we demonstrated that saquinavir had no inhibitory action on the hydrolysis of an aspartic peptidase substrate by *Leishmania* extracts, which could be linked to the reduced killing capability of this inhibitor.

The HIV PI nelfinavir did not inhibit the strain of *L. chagasi* obtained from an HIV-positive patient under antiretroviral treatment #2, which has three different aspartic HIV PIs in its composition, unlike treatment #1, which is composed exclusively of reverse transcriptase inhibitors and a disulphide reagent (Table 1). One of the major problems in the treatment of leishmaniasis is the emergence of resistance to current chemotherapeutics. Indeed, it is known that microorganisms can acquire resistance rapidly and a concern in HIV/*Leishmania*-coinfected patients is the emergence of *Leishmania* resistance.² It is possible that this treatment regimen with HIV PIs in coinfected patients could induce parasite resistance to this drug class.

Previous studies have reported a dose-dependent inhibition of aspartic peptidase activity by HIV PIs.^{9,10} These findings suggest that an aspartic peptidase may be the intracellular target of the HIV PIs.^{9,10} Indeed, an indirect approach confirms this hypothesis. A *Saccharomyces cerevisiae* knockout for *ddi1*, an

orthologue of *Leishmania* aspartic peptidase, was functionally complemented with the *Leishmania* orthologue, reverting the phenotype to the wild type. This phenotype reversion was also induced by HIV PIs.²⁸ Also, HIV PIs can directly inhibit the proteolytic activity responsible for degrading aspartic peptidase substrates.^{10,11} In this context, it is interesting to note that the parasites isolated from a patient under treatment with HIV PIs presented considerably less aspartic peptidase activity than isolates from patients who were untreated or treated only with reverse transcriptase inhibitors. When the strain subjected to HIV PI pressure *in vivo* was exposed to successive *in vitro* passages with nelfinavir, it revealed a significant decrease in the overall aspartic peptidase activity. The selective pressure of drugs can induce the blockage or loss of virulence factors in parasites, as already shown in fungi with respect to HIV PIs. For example, indinavir interfered with polysaccharide capsule formation in *Cryptococcus neoformans*, the major virulence factor produced by this opportunistic fungal pathogen, and it is able to inhibit the hydrolytic activities of secreted aspartic-type peptidases from *Fonsecaea pedrosoi* conidial and mycelial cells, virulence factors capable of interfering with host defence mechanisms by affecting the integrity of relevant host proteins.^{29–31} It is possible that the *Leishmania* species obtained from patients under antiretroviral treatment had undergone a metabolic change that resulted in differential expression of their virulence factors, which affects parasite susceptibility to HIV PIs. It should be pointed out that the inhibitory effects of HIV PIs against *Leishmania in vitro* are in the micromolar range, much higher than those needed for HIV peptidase inhibition (nanomolar range). Although the concentrations required to interfere with intracellular amastigotes are considerably lower than those required for promastigotes,^{10,11,13} they are still higher than those needed for the inhibition of HIV progression in humans. However, we have to consider that HAART consists of a combination of antiretroviral drugs and the pharmacodynamics of an *in vitro* model are very different from those in humans. Curiously, HIV PIs seemed to have only a marginal effect on a murine infection with *L. amazonensis*.³²

No screening study of combination therapy for *Leishmania*/HIV coinfection has been tested in a controlled trial to date. Since repeated exposure to single chemotherapy for leishmaniasis facilitates the emergence of parasite resistance, combination therapy might be particularly relevant to the treatment of coinfected patients and maintenance of susceptibility to treatment in patients who experience disease relapses.² Whereas HIV PIs have a broad spectrum of action, having effects on diverse species of *Leishmania*, and because the current standard therapy for leishmaniasis shows serious side effects and pentavalent antimonials and amphotericin B are more toxic to HIV patients, combination therapy comprising HIV inhibitors and current chemotherapy for leishmaniasis might provide increased efficacy of both therapies, such as reduction of side effects, shorter treatment time and reduced costs.

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Transparency declarations

None to declare.

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