Intracellular Survival of *Leishmania* Species That Cause Visceral Leishmaniasis Is Significantly Reduced by HIV-1 Protease Inhibitors

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Visceral leishmaniasis is now recognized as an opportunistic disease in individuals infected with human immunodeficiency virus type 1 (HIV-1). Although the usefulness of HIV-1 protease inhibitors (PIs) in antiretroviral regimens is well documented, little is known about their potential impact in the setting of *Leishmania*/HIV-1 coinfections. We now report that, although selected PIs do not inhibit the growth of *Leishmania infantum* promastigotes alone in culture, these drugs significantly inhibit the intracellular survival of parasites in phorbol myristate acetate–differentiated THP-1 macrophages and human primary monocyte-derived macrophages (MDMs). Furthermore, a field isolate of *Leishmania donovani* resistant to sodium stibogluconate (SbV), one of the drugs most commonly used to treat leishmaniasis, is equally susceptible to the tested PIs compared with a sensitive strain, thus suggesting that resistance to SbV does not result in cross-resistance to PIs. Importantly, the efficacy of PIs to reduce the intracellular growth of *Leishmania* parasites is also observed in MDMs coinfected with HIV-1.

In the wake of the extensive and rapid expansion of the worldwide AIDS pandemic, several infectious diseases have developed into AIDS-associated opportunistic infections. This phenomenon complicates the therapeutic measures aimed at controlling HIV-1, the causative agent of AIDS, and the various opportunistic pathogens seen in the context of coinfections. Data from retrospective and prospective surveillance studies indicate that AIDS and leishmaniasis, which is caused by intracellular protozoan parasites of the genus *Leishmania*, overlap in several subtropical and tropical regions around the world. Interestingly, the number of *Leishmania*/HIV-1 coinfections is increasing even in the Mediterranean basin. Leishmaniasis—in particular, visceral leishmaniasis (VL)—is now recognized as an opportunistic disease in immunocompromised patients, including those infected with HIV-1 [1]. In coinfected patients, VL is caused primarily by *Leishmania infantum* and *Leishmania donovani*.

The *Leishmania* parasite exists in 2 distinct morphological forms: the slender flagellated promastigote and the oval intracellular amastigote. Motile promastigotes are transmitted during the blood meal of the sandfly vector and are eventually internalized within tissue phagocytes and phagocytic cells recruited to the site of infection, where they transform into the nonmotile intracellular amastigote form. The fact that both the *Leishmania* parasite and HIV-1 can infect the same target cells (namely, macrophages and dendritic cells) suggests the existence of complex interactions between both pathogens. This scenario is validated by the previous demonstration that *L. infantum* enhances HIV-1 replication in human primary monocyte-derived macrophages (MDMs) [2] and that such cells allow a more important
intracellular growth of the parasite on infection with HIV-1 [3]. In HIV-1–infected patients, greater viral loads are associated with a higher prevalence of *Leishmania* infection, and lower CD4+ T cell counts are linked to VL and disseminated forms of leishmaniasis [4, 5]. Furthermore, HIV-1–infected patients show higher amastigote counts in their peripheral circulation than do HIV-1–negative individuals [6, 7].

Highly active antiretroviral therapy (HAART) has been shown to significantly improve the prognosis of patients infected with HIV-1. This aggressive therapeutic strategy is composed of either nonnucleoside reverse-transcriptase inhibitors or protease inhibitors (PIs), in combination with at least 2 nucleoside reverse-transcriptase inhibitors. PIs block the active site of aspartyl protease, a viral enzyme in HIV-1 essential for the maturation of viral proteins, by mimicking endogenous peptides (reviewed in [8]). Although HAART is highly effective at suppressing systemic viral load, there is a paucity of data on its possible impact in the context of coinfections. It has been demonstrated that HIV-1 PIs are active in vitro against *Plasmodium* organisms at clinically achievable concentrations [9] and are active in murine malaria models [10]. Moreover, a synergistic effect of PIs and chloroquine against *Plasmodium falciparum* has been reported [11]. It should be noted that the incidence of *Leishmania*/HIV-1 coinfections has been reduced since the introduction of HAART [12]. Interestingly, a recent study described a dose-dependent leishmanicidal activity in vitro after treatment of *L. infantum* and *Leishmania major* promastigotes with 2 different PIs (indinavir [IDV] and saquinavir [SQV]) [13]. However, the sensitivity of *Leishmania* parasites to PIs has yet to be evaluated in an infection model.

In the present work, we analyzed the efficacy of 3 HIV-1 PIs (nelfinavir [NFV], ritonavir [RTV], and SQV) in controlling the intracellular growth of *Leishmania* parasites in the human monocytic cell line THP-1 and, more importantly, in human primary MDMs. We show here that the intracellular survival of both *L. infantum* and *L. donovani* is markedly reduced after treatment with PIs. The antileishmanial activity of PIs was still seen even when infection was done with a *Leishmania* field isolate that is resistant to sodium stibogluconate (SbV), the drug most commonly used for the treatment of leishmaniasis. In addition, we show that replication of both *Leishmania* parasites and HIV-1 is inhibited in coinfected MDMs treated with a PI.

**METHODS**

**Cell culture.** *L. infantum* (MHOM/MA/67/ITMAP-263) promastigotes transfected with pGLα–NEOαLUC1.2 (hereafter, Li-luc) [14] were maintained at pH 7.0 and a temperature of 25°C. Promastigotes were cultured in RPMI 1640 medium (Wisent) supplemented with 10% fetal bovine serum (FBS), 5 μg/mL hemin (Sigma), 25 μmol/L HEPES, 2 mmol/L NaHCO₃, and 80 μg/mL G418 (a selective agent; Invitrogen) in 25-cm² flasks. Li-luc axenic amastigotes were obtained by seeding Li-luc promastigotes in 25-cm² flasks containing MAA/20 medium at a final pH of 5.6 supplemented with 80 μg/mL G418. MAA/20 consists of modified medium 199 (Gibco BRL) with Hank's salts, supplemented with 0.5% soybean tryptose (Pasteur Diagnostics), 15 mmol/L D-glucose, 5 mmol/L L-glutamine, 4 mmol/L NaHCO₃, 0.023 mmol/L bovine hemin, and 25 mmol/L HEPES at a final pH of 6.5 and supplemented with 20% of FBS, as described elsewhere [15]. These axenic amastigotes show morphological, biochemical, and biological characteristics similar to those of amastigotes isolated in vivo (data not shown).

The *L. donovani* field strains 9518 and 9551 were isolated between 1995 and 1998 in the Muzzarphapur district of Bihar, India, from patients in whom kala-azar had been parasitologically diagnosed by microscopic examination of splenic aspirate smears [16, 17]. It has been shown that 9518 (hereafter, Ld 9518-luc) is resistant to the antileishmanial drug SbV, whereas 9551 (hereafter, Ld 9551-luc) is sensitive to SbV. Ld 9518-luc and Ld 9551-luc, stably transfected with the GL-αNEOαLUC1.2 vector, were maintained in the same medium as Li-luc, except for the addition of 10 μmol/L biopterin (Sigma).

THP-1 cells were plated in 24-well plates (2 × 10⁴ cells/well) and allowed to differentiate into macrophages by a 48-h treatment with 20 ng/mL phorbol myristate acetate (PMA; Sigma) before their use in infection assays. Human monocytes were purified by adherence to plastic in RPMI 1640 supplemented with 5% autologous human serum. Next, monocytes were allowed to differentiate into MDMs in complete RPMI 1640 supplemented with human recombinant macrophage colony-stimulating factor (100 ng/mL) for 7 days. MDMs were plated in 24-well plates (2 × 10⁵ cells/well) for 24 h before *Leishmania* infection and in 96-well plates (5 × 10⁴/well) for the toxicity assays.

**Toxicity assays.** NFV, RTV, and SQV, obtained through the National Institutes of Health AIDS Repository Reagent Program, were resuspended at a concentration of 50 mmol/L in dimethyl sulfoxide (DMSO), for a maximum final concentration in culture of 0.1% DMSO. Motile promastigotes were counted using a hemocytometer and were resuspended at a final concentration of 5 × 10⁵ parasites/mL in 5 mL of culture medium. Each PI was added to the culture at a final concentration of 12.5 or 25 μmol/L. Appropriate dilutions of DMSO, corresponding to those used to prepare the drug solutions, were used as controls. Live parasite counts were determined by trypan blue exclusion using bright-field phase-contrast microscopy after a 72-h incubation at 25°C. Axenic amastigotes were counted as described above and resuspended at a final concentration of 1 × 10⁶ parasites/mL in 5 mL of MAA/20 medium. After a 72-h incubation with the tested PIs or the control solutions, the number of intact amastigotes was assessed using trypan blue exclusion. PMA-differentiated THP-1 macrophages or MDMs were
first plated in 96-well plates (5 x 10^4 cells/well) before exposure to the studied PIs or control solutions. Cells were incubated for 72 h at 37°C in a 5% CO₂ atmosphere. Next, cells were washed extensively with PBS before viability was measured by MTS assay, in accordance with the manufacturer's instructions (CellTiter96AQ nonradioactive assay; Promega). In this colorimetric method, metabolically active cells convert the MTS reagent into water-soluble formazan that absorbs light at an optical density of 490 nm.

Infection assays. PMA-differentiated THP-1 macrophages or MDMs (2 x 10^5 cells/well in a 24-well plate) were washed with PBS and then infected with stationary-phase Li-luc, Ld 9518-luc, or Ld 9551-luc promastigotes (7-day culture) at a parasite to cell ratio of 15:1 or Li-luc axenic amastigotes (4-day culture) at a parasite to cell ratio of 3:1, for 2 h at 37°C in a 5% CO₂ atmosphere. Noninternalized parasites were eliminated by 3 extensive washes with PBS. Thereafter, Leishmania-infected cells were either left untreated or treated for 72 h at 37°C with PIs. Controls consisted of dilutions of dimethyl sulfoxide (DMSO) corresponding to those used to prepare the drug solutions. Next, cells were washed 3 times with PBS and lysed, and luciferase activity was measured as described elsewhere, using a luminometer (MLX; Dynex Technologies) [14]. The experiments were performed in triplicate. In coinfection experiments, MDMs (1.5 x 10^5 cells/well in a 48-well plate) were first incubated with stationary-phase Li-luc promastigotes (7-day culture) at a parasite to cell ratio of 10:1 for 3 h. Noninternalized parasites were washed out with warm PBS. Infected MDMs were then pulsed with fully competent R5-tropic virions (i.e., NL4–3BalEnv) (10 ng of p24 per 1 x 10^5 cells) for 2 h, washed, and maintained in drug-free medium for 72 h, to establish infection. Coinfected MDMs were treated with the indicated PIs at 12.5 or 25 μmol/L or with the appropriate controls for 72 h. Leishmania growth was estimated by measuring luciferase activity, whereas HIV-1 production was assayed by ELISA, measuring the p24 content in cell-free supernatants, as described elsewhere [18].

Statistical analyses. The statistical significance of the results was defined by performing a 1-way analysis of variance with Bonferroni's posttest to compare treated versus control samples. All statistical analyses were performed using Prism software (version 3.03). Differences were considered statistically significant at P < .05.
RESULTS

Our experiments were performed with the closely related species *L. infantum* and *L. donovani* because they both cause VL, a major tropical disease that has emerged as an important opportunistic infection among patients with AIDS. We tested 3 first-generation PIs, namely, NFV, RTV, and SQV. A number of experiments were performed using the THP-1 cell line, a human acute monocytic leukemia cell line that has been used for years to study *Leishmania* infections. Although immortal myeloid cell lines are considered useful experimental tools to decipher the complexity of interactions between *Leishmania* parasites and cells of the myeloid lineage, experiments were also conducted in human primary MDMs to parallel natural conditions more closely.

**Susceptibility to PIs of Leishmania promastigotes and axenic amastigotes cultured in vitro.** It has recently been reported that IDV and SQV demonstrate a dose-dependent leishmanicidal activity, which was more important for *L. major* (a species that causes cutaneous leishmaniasis) than for *L. infantum* promastigotes [13]. Our initial series of investigations were thus aimed at assessing the direct effect of PIs on cultured *Leishmania* promastigotes, the insect vector form of the parasite. In contrast to the observations made by Savoia et al. [13], there was no growth inhibition of Li-luc promastigotes after treatment with the 3 PIs tested (figure 1A). Although the growth of extracellular Li-luc amastigotes was not affected by SQV, we found that NFV and RTV significantly inhibited growth of this parasite population (figure 1B). For example, 77% growth inhibition was achieved with 25 μmol/L NFV, compared with 83% for a similar dose of RTV.

**Leishmanicidal potency of PIs against the intracellular growth of Leishmania parasites.** Given that PIs can reduce the growth of cultured *Leishmania* amastigotes, we monitored the action of PIs on the intracellular survival of this parasite form. The toxicity of PIs was first evaluated in the 2 experimental cell systems tested (i.e., PMA-differentiated THP-1 macrophages and human primary MDMs). A slight deleterious effect was seen in THP-1 cells with a 25 μmol/L concentration of NFV, and a more dramatic toxicity was detected when a similar concentration of SQV was used (figure 2A). However, no toxicity...
Table 1. Susceptibility of intracellular parasites to protease inhibitors (PIs) in phorbol myristate acetate–differentiated THP-1 macrophages and monocyte-derived macrophages (MDMs).

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<tr>
<th>PI, concentration</th>
<th>Percentage of inhibition</th>
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<tr>
<td></td>
<td>THP-1 + promastigotes</td>
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<td>Nelfinavir</td>
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<tr>
<td>Saquinavir</td>
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<td>48.5</td>
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<td>25.0 μmol/L</td>
<td>92.3</td>
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**NOTE.** Data are the percentage of inhibition of infection with *Leishmania* parasites in the presence of PIs, compared with that in samples treated with the diluent (dimethyl sulfoxide), and are reported as the means of 3 independent experiments.

The antileishmanial potency of PIs was finally tested against parasites growing inside human mononuclear phagocytes (the natural host of *Leishmania* parasites), using PMA-differentiated THP-1 macrophages and MDMs, 2 established in vitro models of *Leishmania* infection. All PIs studied exerted pronounced effects against the intracellular parasites in our in vitro infection cell systems (table 1). For example, NFV acted as a powerful inhibitor of the intracellular growth of Li-luc promastigotes, with 79.4% inhibition in THP-1 macrophages (*P < .001*) and a 71.3% decrease in MDMs (*P < .001*) when used at a final concentration of 12.5 μmol/L. Significant leishmanicidal potency by the 3 PIs tested was also observed when THP-1 macrophages and MDMs were infected with Li-luc axenic amastigotes. NFV at a final dose of 25 μmol/L was the most effective compound, leading to 79.9% inhibition in the intracellular survival of parasites, compared with 43.7% and 61.4% for RTV and SQV, respectively.

Next, we evaluated the efficacy of PIs to limit the intracellular growth of a field isolate of *L. donovani* resistant to SbV (i.e., Ld 9518-luc). The data shown in table 2 indicate that PIs can limit the intracellular growth of both SbV-sensitive and SbV-resistant clinical isolates of *L. donovani* to a similar extent and in a dose-dependent manner.

**Effect of PIs on the intracellular survival of Leishmania in MDMs coinfected with HIV-1.** The rising incidence of *Leishmania*/HIV-1 coинфекtion prompted us to monitor the impact of PIs on *Leishmania* growth inhibition in the context of coinfection with HIV-1. As expected, virus replication was totally abolished in the presence of PIs in MDMs coinfected with *Leishmania* parasites and HIV-1 (figure 3). Interestingly, the antileishmanial activity of PIs in human primary MDMs was not diminished in the presence of HIV-1 (table 3).

**Table 2. Susceptibility of Ld 9551-luc and Ld 9518-luc to protease inhibitors (PIs) in monocyte-derived macrophages.**

<table>
<thead>
<tr>
<th>PI, concentration</th>
<th>Percentage of inhibition</th>
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<tr>
<td></td>
<td>Ld 9551-luc promastigotes</td>
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<td>25.0 μmol/L</td>
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**NOTE.** Data are the percentage of inhibition of infection with *Leishmania* parasites in the presence of PIs, compared with that in samples treated with the diluent (dimethyl sulfoxide), and are reported as the means of 3 independent experiments. NT, not tested.
Figure 3. Effect of protease inhibitors (PIs) on HIV-1 production in coinfected monocyte-derived macrophages (MDMs). First, MDMs were inoculated with stationary-phase promastigotes of Li-luc for 3 h at a parasite to cell ratio of 10:1. Next, MDMs were infected with replication-competent HIV-1 particles (i.e., NL4-3BalEnv) for 2 h. Coinfected MDMs were left untreated for 72 h, PIs were added at the indicated concentrations, and cells were incubated for another 72 h. Controls consisted of dilutions of dimethyl sulfoxide (DMSO) corresponding to those used to prepare the drug solutions. Parasite growth was estimated by measuring luciferase activity, and virus replication was assessed by quantifying the p24 content (p24 values under the lower detection limit of the ELISA [0.3125 pg/mL] are indicated by $). NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir.

minor activity against L. infantum [13]. However, in our hands, no significant toxicity was observed with NFV, RTV, or SQV toward promastigotes in culture. Differences in experimental methods may account for the conflicting results (e.g., Leishmania species, source and purity of PIs, media, and final concentrations of DMSO). However, we did observe a potent PI-mediated antileishmanial activity in axenic amastigotes with subcytotoxic concentrations of NFV and RTV. Although the antiretroviral PIs were developed to be specific for the HIV-1 aspartyl protease, it is possible that they may additionally target a Leishmania enzyme that has yet to be identified. The importance of proteases in the Leishmania life cycle is illustrated by the fact that their replication is prevented by lactacystin, a specific inhibitor of proteasomes [21]. Proteasomes are involved in protozoan differentiation and replication, in addition to abnormal protein degradation. Interestingly, amastigotes developing within target cells during human infection are more sensitive to lactacystin than promastigotes developing in culture medium [21].

Given this context, we evaluated the effect of HIV-1 PIs on the intracellular growth of Leishmania located inside target cells. It has indeed been suggested that it does not suffice to test drugs for antileishmanial activity against promastigotes alone, because the drugs might act differently on amastigotes in an intracellular environment [22]. When we first assayed the direct toxicity of PIs on human phagocytic cells, we observed some toxic effect of NFV and SQV on the THP-1 cell line, whereas no toxicity was found in MDMs with all PIs tested. Moreover, inhibition of Leishmania infection was higher in THP-1 cells treated with RTV or SQV than in similarly treated MDMs. For SQV, this may be partially due to the slightly higher toxic effects of the drug on the cell line.

Although extracellular axenic amastigotes were somewhat susceptible to the PIs, their intracellular survival in the presence of PIs was similar to that of intracellular promastigotes, suggesting that PIs affect the intracellular parasites primarily at the amastigote stage. This is based on the previous finding that the developmental switch between promastigotes and amastigotes occurs rapidly once Leishmania promastigotes are inside parasitophorous vacuoles [23]. In coinfection experiments with Leishmania and HIV-1, the PIs were added 3 days after infection, suggesting again that PIs exert their effect mainly on amastigotes by increasing parasite killing and/or slowing down their replication. It is thus possible that the target of PIs is expressed preferentially in the amastigote stage of the parasite. Moreover, the PIs can modulate the intracellular survival of Leishmania parasites by affecting some basic functions of the macrophage. For example, the HIV-1 PIs can interfere with the process of parasitophorous vacuole formation.

We are currently investigating the possibility that PIs could interfere with phagosome/lysosome biogenesis and/or parasite phagocytosis, replication, and killing. Another possible explanation for why extracellular cultured parasites are less susceptible to PIs than parasites growing inside target cells is the idea that cells of the macrophage lineage can concentrate higher levels of drugs than extracellular axenic amastigotes. Studies with radioactive drugs are needed to resolve this issue.

Table 3. Susceptibility of intracellular parasites to protease inhibitors (PIs) in monocyte-derived macrophages coinfecte...
We found that there was variability in the activity of the studied PIs against the different parasite forms (e.g., extracellular amastigotes and intracellular amastigotes). Interestingly, differential drug susceptibility in the 2 distinct morphological forms of *Leishmania* parasites has already been reported for antileishmanial compounds. Indeed, it has been shown elsewhere that amastigotes are more susceptible to SbV than promastigotes [24]. It was proposed that the route of entry of SbV and the active trivalent form of the metal are different in promastigote and amastigote stages [25]. It can thus be postulated that accumulation of the tested HIV-1 PIs in extracellular amastigotes versus intracellular amastigotes takes place through distinct uptake systems.

The emergence of a resistance phenotype to pentavalent antimonials represents one of the most serious problems in the control of VL, especially in areas such as the state of Bihar in India [26]. We thus tested the effect of PIs in MDMs infected with SbV-sensitive (i.e., Ld 9551-luc) and SbV-resistant (i.e., Ld 9518-luc) *Leishmania* isolates. Our results show that PIs are highly efficient at diminishing the intracellular survival of the *Leishmania* field isolate resistant to SbV, therefore suggesting that resistance to SbV does not result in cross-resistance to PIs. In India, several SbV-resistant *Leishmania* strains have been shown to overexpress the multiresistance gene MRPA [27] (formerly known as PGPA [28]). However, that is not the case for the SbV-resistant *Leishmania* strain used in this study [17]. Because PIs are substrates for P-glycoprotein and multidrug resistance protein 1 in human cells [29, 30], it would be interesting to evaluate whether *Leishmania* strains that overexpress MRPA might display a lower sensitivity to PIs. In this context, Jones et al. [31] evaluated the intracellular accumulation of HIV-1 PIs and showed that CEM clones overexpressing P-glycoproteins exhibit significantly reduced drug accumulation. Moreover, they demonstrate that NFV, the most efficient PI in our study, accumulated more substantially than did SQV and RTV.

PIs were designed to inhibit the HIV-1 aspartyl protease. One could hypothesize that, when used in persons infected with both *Leishmania* parasites and HIV-1, the effect of PIs on leishmanial infection would be reduced. This postulate is based on the idea that PIs will preferentially interact with the HIV-1 aspartyl protease because of its high binding affinity compared with other putative target(s) present either in the parasite itself or within target cells. However, our results show that PIs diminish infection with *Leishmania* parasites to the same extent when HIV-1 is also present. Other studies have revealed that this class of drugs has adverse effects, such as inflammatory activity [32], blockade of the cell proteasome function [33], and induction of apoptosis [34]. PIs can also affect the phagocytosis of parasites, as these compounds have been shown to impair the nonopsonic phagocytosis of *P. falciparum*–parasitized erythrocytes by human macrophages [35]. In contrast to the effects observed in malaria, a reduction in *Leishmania* phagocytosis would be protective for the patients, because parasites would be limited in finding a safe place to multiply.

Taken together, our results shed light on a new and unexpected treatment opportunity for leishmaniasis and also on the need to take care when treating patients who are coinfected with *Leishmania* parasites and HIV-1. Additional studies are urgently needed to evaluate the therapeutic efficacy of a class of drugs intended for the treatment of one disease (i.e., PIs to control HIV-1 load) in a parasitic disease frequently diagnosed in HIV-1–infected persons (i.e., leishmaniasis). The present study underscores the complexity of managing opportunistic infections of pathological importance in individuals carrying HIV-1.

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References


