HIV Protease Inhibitors Inhibit the Development of Preerythrocytic-Stage Plasmodium Parasites

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Recent studies have demonstrated that human immunodeficiency virus (HIV) protease inhibitors (PIs) exert inhibitory effects on erythrocytic stages of the human-malaria parasite Plasmodium falciparum in vitro and on erythrocytic stages of the rodent-malaria parasite Plasmodium chabaudi in vivo. Although it remains unclear how HIV PIs inhibit the parasite, the effect seen on parasite development in the erythrocytic stages is potent. The effect on preerythrocytic stages has not yet been investigated. Using the rodent parasite Plasmodium berghei, we screened a panel of HIV PIs in vitro for effects on the preerythrocytic stages. Our data indicated that the HIV PIs lopinavir and saquinavir affect preerythrocytic-stage parasite development in vitro. We then evaluated the effect of HIV PIs on preerythrocytic stages in vivo using the rodent parasite Plasmodium yoelii. We found that lopinavir/ritonavir had a dose-dependent effect on liver-stage parasite development. Given that sub-Saharan Africa is where the HIV/AIDS pandemic intersects with malaria, these results merit analysis in clinical settings.

The World Health Organization estimates that 300–500 million cases of malaria and 1–2 million deaths due to malaria occur annually, with most malaria-related morbidity and mortality occurring in children [1]. This malarial disease burden is compounded by the pandemic of HIV/AIDS, which is most prevalent in areas in which malaria is endemic, notably sub-Saharan Africa [2–4]. Moreover, when HIV and a malaria parasite are present as a coinfection, they enhance each other’s pathogenicity [5, 6]. Clinical studies have shown that HIV burden is higher in patients with malaria than in control subjects without malaria [6–8]. It has even been hypothesized that the increased viral loads seen in HIV-positive patients with malaria may be an important factor in fueling the spread of HIV in sub-Saharan Africa [9]. Moreover, HIV-infected individuals with malaria are more likely to have recrudescence of malaria [10]. HIV affects the acquisition of malaria-specific immunity in individuals living in areas of higher and more stable transmission intensity, and this is especially true in patients with lower CD4+ cell counts and higher viral loads [11, 12]. In the absence of an effective antimalarial vaccine and with the intersection of the HIV/AIDS pandemic and malaria epidemics, the need to optimize treatment of patients with HIV infection while reducing the malarial disease burden becomes clearly urgent.

The 3 malarial life-cycle stages targeted for treatment and prevention strategies are the preerythrocytic stages (sporozoite stage and exoerythrocytic forms [EEFs]), the asexual blood stages, and the transmission stages (sexual blood stage and mosquito stage). Sporozoites are the infective form of the parasite and are injected into the mammalian host during blood feeding by infected Anopheles mosquitoes. From the injection site, sporozoites go to the liver, where they invade hepatocytes and develop into EEFs. Mature EEFs contain merozoites that exit the hepatocyte and invade erythrocytes, initiating the erythrocytic stages of disease [13].
Most antimalarial drugs being investigated or developed for *Plasmodium* species target the asexual blood stages, which are responsible for the clinical symptoms of disease. However, intervention during the preerythrocytic stages offers the ability to target the parasite while it is present in low numbers and before it initiates blood-stage infection [14].

Previous work demonstrated that HIV protease inhibitors (PIs) exert a potent antiplasmodial effect on erythrocytic-stage parasites [15–19]. Other classes of antiretrovirals tested for antimalarial effect, such as nonnucleoside reverse-transcriptase inhibitors, have not shown similar efficacy in vitro against *Plasmodium falciparum* erythrocytic-stage parasites [15, 19, 20]. Evaluation of the effect of HIV PIs on preerythrocytic stages has heretofore not been performed. Thus, using rodent models of malaria, we investigated the effect of HIV PIs on the preerythrocytic-stage parasite.

**METHODS**

**Mice.** Female Swiss Webster mice, aged 4–6 weeks and weighing 20–25 g, were purchased from Taconic and were handled according to institutional guidelines.

**Parasites and mosquitoes.** *Plasmodium berghei* (NK65 and ANKA strains) and *Plasmodium yoelii* (17XNL) sporozoites were grown in *Anopheles stephensi* mosquitoes. Three to 5 days after emergence, mosquitoes were fed on *P. yoelii*-infected or *P. berghei*-infected Swiss Webster mice with abundant gametocyte-stage parasites. Salivary gland sporozoites were harvested by mosquito dissection on days 14 and 15 after the infective blood meal for *P. yoelii* and on days 18–20 after the infective blood meal for *P. berghei*. Sporozoites were counted using a hemocytometer.

**Drug preparation.** For in vitro assays, pure HIV PIs were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Stocks (×1000) were prepared in DMSO as described elsewhere [16]. Final concentrations for experiments were prepared by diluting stock with culture medium. For all in vitro experiments, DMSO diluted in culture medium alone was added to the control wells (the DMSO concentration was ≈0.04% per well, a concentration equal to that added to treated wells).

For in vivo assays, pure saquinavir-mesylate (provided by Hoffmann-LaRoche) was prepared from stock solutions diluted in olive oil. Olive oil was used as a vehicle and control for the saquinavir experiments (high-fat, high-calorie meals enhance the pharmacokinetic profile of saquinavir [21]). Given the variability in store-bought olive oil, we maintained the same stock bottle for the duration of experimentation, and we used the same stock for control and treated animals. Only unboosted saquinavir was tested in vivo. Lopinavir/ritonavir and control vehicle (provided by Abbott Laboratories) were prepared using an optimized ratio of lopinavir to ritonavir (2:1) that was based on preclinical rodent pharmacokinetic data from Abbott [22].

**Cytotoxicity assay.** Hepa 1-6 cells (CRL-1830; ATCC) were grown at 37°C in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum and 1% l-glutamine (culture medium). Cells were plated at a density of 2.5 × 10⁵ cells/well in Labtek chamber slides and cultured for 24 h. Cells were then incubated for 48 h in culture medium containing the appropriate drug concentration. The drug-containing medium was changed twice per day. At 24 and 48 h, cells were visualized for morphology with a standard light microscope. At 48 h, cells were washed with PBS, treated with trypsin, and counted using a hemocytometer.

**Microscopic assay for quantification of EEF development in vitro.** Hepa 1-6 cells were plated as outlined above, and 1 × 10⁵ *P. berghei* sporozoites were added to each well. Parasites were allowed to invade the Hepa 1-6 cells for 1 h, after which unattached sporozoites were washed away. HIV PI stock solutions, diluted in culture medium to the doses indicated in figure 1, were added to cells 1 h after the addition of sporozoites. After 46–48 h, cells were fixed with cold methanol, washed, and stained with monoclonal antibody (MAb) 2E6, which recognizes *Plasmodium* hsp70 [23], followed by anti–mouse immunoglobulin conjugated to rhodamine. Cell nuclei were stained with 1 μg/mL 3,3’-diaminobenzidine (Sigma). EEFs were counted using a Nikon Eclipse E600 fluorescence microscope; counting was done in 50 fields per well, and each point was plated in triplicate. Five randomly selected EEFs from each of 3 triplicate wells (with a total of 15 EEFs per assay per condition) were used to determine the average EEF size. The widest diameter of the EEF was measured using a Leica inverted laser-scanning confocal microscope (model TCS SP2 AOBS) with Leica LCSlite software (version 2.61, build 1538). Measurements were used to calculate means ± SDs. All in vitro development assays were performed with unboosted PIs (i.e., in the absence of ritonavir). Each experimental condition was run in triplicate. Differences between the mean number of EEFs per 20 high-powered fields in treated and control wells as well as differences in mean size (estimated by the widest diameter measurement) in treated and control wells were analyzed for statistical significance.

**Microscopic assay for quantification of sporozoite invasion in vitro.** To determine effect of HIV PIs on sporozoite invasion, a double-staining assay that differentiates between intracellular and extracellular sporozoites was performed as described elsewhere, with a few modifications [24, 25]. The assay was performed using Hepa 1-6 cells, MAb 3D11 (antibody directed against the repeat region of circumsporozoite protein, the major sporozoite surface protein) [26], and secondary antibodies as detailed below. Hepa 1-6 cells were plated as outlined above, and *P. berghei* sporozoites, pretreated with 40 μmol/L saquinavir or 40 μmol/L lopinavir in culture medium for 30 min at 27°C, were washed and added to the cells. Control sporozoites were pretreated with 0.04% DMSO alone. Parasites were incubated with Hepa 1-6 cells for
1 h, after which unattached sporozoites were removed. Cells were then fixed with 4% paraformaldehyde and incubated with MAb 3D11 followed by goat anti–mouse immunoglobulin conjugated to fluorescein isothiocyanate (FITC). Cells were then permeabilized with methanol and incubated with MAb 3D11 followed by goat anti–mouse immunoglobulin conjugated to rhodamine. The number of extracellular sporozoites (FITC labeled) and total sporozoites (rhodamine labeled) per field were counted in 50 fields per well. Percent invasion was then calculated. All in vitro invasion assays were performed with unboosted PIs. The mean number of parasites invading (in treated and control wells run in triplicate for each experimental condition) was calculated and analyzed for statistically significant differences.

**Assay for quantification of P. yoelii liver-stage development in vivo.** P. yoelii was used for in vivo challenge because it is more infectious than *P. berghei* in vivo and better models the infectivity of the human-malaria parasite *P. falciparum* [27, 28]. The doses of HIV PIs indicated below were administered to mice by gavage. For once-a-day (QD) dosing regimens, doses were administered to mice 6 h before sporozoite inoculation, with 1 additional dose given 24 h later. For twice-a-day (BID) dosing regimens, doses were administered 6 h before sporozoite inoculation and every 12 h after the first dose for 2 more doses. Forty hours after sporozoite inoculation, mice were killed, livers were harvested, and total RNA was isolated using TRI reagent (Molecular Research Center), in accordance with the manufacturer’s instructions. Parasite burden in the liver was quantified as outlined elsewhere [29]. Briefly, reverse transcription was performed using 1 µg of total RNA and random hexamers. After this, real-time polymerase chain reaction (PCR) was performed using primers that recognize *P. yoelii*–specific sequences within the 18S rRNA and the QuantiTect SYBR Green PCR Kit (Qiagen). Ten-fold dilutions of a plasmid construct containing the *P. yoelii* 18S rRNA gene were used to create a standard curve. Differences between the mean amounts of parasite 18S rRNA in the
livers of treated and control mice were analyzed for statistical significance.

Assay for prepatent period (or assay for detection of parasites in blood). This assay was performed as described elsewhere [30], with a few modifications. To evaluate whether the development of EEFs was fully arrested with the HIV PI dosing regimens used, mice were administered 100 mg/kg lopinavir with 50 mg/kg ritonavir and then challenged with 1/1000102 sporozoites. Mice were injected intravenously with 1/1000102 P. yoelii sporozoites, and, beginning on day 3 after sporozoite injection, mice were monitored for the presence of erythrocytic-stage parasites by means of Giemsa-stained blood smears.

Pharmacodynamics of lopinavir/ritonavir and saquinavir in vivo. Groups of 5–6 mice were given the BID and QD HIV PI dosing regimens outlined above, and serum samples were obtained 6, 12, and/or 24 h after the last dose was administered. Blood was obtained by submandibular vein sampling and centrifuged; serum was removed, frozen at −80°C, and shipped overnight on dry ice to Abbott Laboratories for analysis. Analysis of plasma levels was performed by high-performance liquid chromatography at Abbott Laboratories.

Statistical analysis. One-way analysis of variance was used to compare mean parasite numbers in both in vitro and in vivo assays, taking into account multiple replicates. Tukey's method was used to identify statistically significant differences at the P < .05 level. Statistical analyses were done using Prism software (version 5; GraphPad).

RESULTS

Assessment of drug-induced cell toxicity for in vitro assays. The maximum concentration of saquinavir, lopinavir, atazanavir, and amprenavir added to Hepa 1-6 cells at which cell growth was preserved was 40 μmol/L; for nelfinavir, this concentration was 10 μmol/L. At these concentrations, cell counts in control versus treated wells were not statistically significantly different, and morphology in drug-treated parasites (as assessed by light microscopy) was not different from the control (data not shown).

Inhibition of the development of P. berghei EEFs in vitro by saquinavir and lopinavir but not by atazanavir, amprenavir, and nelfinavir. Saquinavir treatment at 10 and 40 μmol/L reduced the number of EEFs by an average of 55% (P < .05) and 65% (P < .05), respectively. Lopinavir treatment at 10 and 40 μmol/L reduced the number of EEFs by an average of 38% (P < .05) and 90% (P < .05), respectively. No significant differences between treated and untreated samples were observed with atazanavir, amprenavir, or nelfinavir (figure 1). EEFs treated with saquinavir or lopinavir at 10 μmol/L were not significantly smaller than control EEFs, but treatment at 40 μmol/L resulted in a reduction in EEF size by an average of 42% for saquinavir and 75% for lopinavir, compared with control.

Inhibition of P. berghei invasion in vitro only by high concentrations of saquinavir. Using P. berghei sporozoites...
preincubated with the PIs, we tested the effect of lopinavir and saquinavir on sporozoite invasion of Hepa 1-6 cells. We did not find an effect with lopinavir at 40 μmol/L, and saquinavir at 40 μmol/L had only a mild effect, with an average percent inhibition of invasion of 28% (*P < .05) (figure 4).

Inhibition of *P. yoelii* development in vivo by lopinavir/ritonavir in a dose-dependent fashion. Lopinavir/ritonavir exerted a dose-dependent effect in reducing liver parasite burden. When mice were administered 25 mg/kg lopinavir with 12.5 mg/kg ritonavir or 100 mg/kg lopinavir with 50 mg/kg ritonavir 6 h before infection and then again 18 h after sporozoite inoculation, parasite liver burden was reduced by 45% and 93%, respectively (figure 5). With these regimens, drug was undetectable in serum 12 h after administration.

Prolongation of the prepatent period by lopinavir/ritonavir. Treated mice had detectable parasites in blood 1–1.3 days later than control mice (table 1).

Lower serum levels of lopinavir/ritonavir in rodent serum sampling reduces liver parasite burden compared with levels achieved in humans receiving standard lopinavir/ritonavir. With BID dosing, 50 mg/kg lopinavir with 25 mg/kg ritonavir produced a mean ± SD peak level of 8.64 ± 1.74 μg/mL (6-h serum level after 3 days of gavage dosing). With the same dose gavaged for 3 days, levels measured 12 h after the last dose had a mean ± SD of 2.7 ± 3.6 μg/mL, and levels measured 24 h after the last dose were undetectable. (For reference, the mean ± SD peak plasma level for HIV-infected adults taking 400 mg of lopinavir with 100 mg of ritonavir BID for 3 weeks is 9.8 ± 3.7 μg/mL, and the mean ± SD steady state trough concentration is 7.1 ± 2.9 μg/mL [Abbott prescribing information]).

For 100 mg/kg lopinavir with 50 mg/kg ritonavir QD and for 50 mg/kg lopinavir with 25 mg/kg ritonavir QD gavaged for 3 days, levels at 12 and 24 h after last dose were all undetectable. (For reference, daily dosing of 800 mg of lopinavir with 200 mg of ritonavir [administered as part of a regimen that included 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate] generates a mean ± SD peak of 1.18 ± 3.7 μg/mL and a mean ± SD trough of 3.2 ± 2.1 μg/mL [Abbott prescribing information]).

All saquinavir levels at 12 h after 3 days of gavage dosing with unboosted doses as high as 200 mg/kg were undetectable.

No inhibition of the development of *P. yoelii* in vivo by saquinavir. We found that saquinavir alone in high doses did not have any effect on the liver stage of the parasite in vivo when administered before infection and during development. We tested doses as high as 200 mg/kg BID and parasite inocula as low as 1 × 10^2 sporozoites but still saw no effect (data not shown).

**DISCUSSION**

We have described an inhibitory effect of HIV PIs on preerythrocytic stages of *Plasmodium* species in rodent models in vitro.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effect of saquinavir (SQ) and lopinavir (LPV) on *Plasmodium berghei* invasion. Sporozoites were preincubated with the indicated concentrations of drug and then added to hepatocytes in the continued presence of the drug for a 1-h period. Extracellular sporozoites and total sporozoites per field were counted in 50 fields per well, and the percent invasion was then calculated. *P < .05* (statistically significant difference between the treated group and the control group).

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Effect of lopinavir/ritonavir on *Plasmodium yoelii* liver burden. Mice were gavaged with lopinavir/ritonavir before being injected intravenously with 10,000 *P. yoelii* sporozoites. Mice were given vehicle (control), 100 mg/kg lopinavir with 50 mg/kg ritonavir once a day (100 mg/kg QD LPV/r), or 25 mg/kg lopinavir with 12.5 mg/kg ritonavir once a day (25 mg/kg QD LPV/r), the lowest dose that had a significant effect. All experiments were performed twice, with 5–6 mice per group per experiment. *P < .05* (statistically significant difference between the treated group and the control group).
and in vivo. For the first time, our data show that lopinavir and saquinavir in vitro as well as lopinavir/ritonavir in vivo have a significant effect on the preerythrocytic development of *Plasmodium* parasites.

Our in vitro data indicate that both lopinavir and saquinavir decreased the number and size of EEFs, and the morphology of drug-treated EEFs was also altered. There was no demonstrable effect on invasion with lopinavir in vitro, even at high concentrations, suggesting that lopinavir works during the intrahepatic development of the parasite.

When tested in vivo, lopinavir/ritonavir significantly reduced liver burden by as much as 55% with a dose as low as 25 mg/kg lopinavir with 12.5 mg/kg ritonavir. Mice treated with lopinavir/ritonavir also tended to show a prolonged time to detectable parasitemia (i.e., a prolonged prepatent period), compared with control mice. This likely reflects the lower liver burden, which was seen in the quantitative PCR experiments. At the doses used, not all parasites were killed, however, because there was eventual detectable blood-stage parasitemia.

The doses of lopinavir/ritonavir used in our experiments are higher than doses used to achieve therapeutic efficacy for HIV treatment in humans, but serum-level analysis showed that the drug levels achieved in our mouse model are comparable to or lower than those achieved in humans receiving standard lopinavir/ritonavir dosing regimens. This is consistent with previous work showing the half-lives and steady-state levels of lopinavir and ritonavir to be shorter in mice than in humans, thus necessitating higher doses of these drugs in rodent models to achieve concentrations that may be compared with levels achieved in patients receiving standard HIV PI dosing [31].

There is an apparent effect with undetectable serum levels of lopinavir/ritonavir. This could be explained by previously published data in rats showing that tissue-to-plasma ratios are highest in organs of the gastrointestinal tract, including the liver, 4 h after oral administration of radioactively-labeled lopinavir/ritonavir [22]. This suggests that, even when plasma levels are low, levels in the liver (the site of parasite development) may be higher and thus may account for the antiparasite effect we observed.

Although we were not able to demonstrate an effect with saquinavir in unboosted form, the question as to whether boosted saquinavir would have an effect remains. Ritonavir, while exhibiting PI activity itself, is often used at subtherapeutic doses to boost the blood levels of other PIs by exploiting its inhibitory effect on liver cytochrome P450 enzymes [31]. Ritonavir use is critical to achieving therapeutic levels of lopinavir in vivo and is less so for saquinavir. Although it is possible that the use of lopinavir or saquinavir with ritonavir produces a synergistic antiparasite effect, it is also possible that the boosting effect of ritonavir enables the HIV PI to maintain therapeutic levels for a longer period of time, thus accounting for its enhanced effect.

Strikingly, our findings parallel previously published findings for an effect of HIV PIs on erythrocytic-stage *Plasmodium* parasites. Lopinavir and saquinavir were previously shown to be the most potent drugs affecting the erythrocytic-stage parasite in vitro with *P. falciparum* (lopinavir had the lowest IC50 for 4 different strains of *P. falciparum*) [16]. Considered together with our present findings, this suggests that HIV PIs may affect a common enzyme or pathway in these distinct *Plasmodium* life-cycle stages.

It remains unclear how HIV PIs function in hindering the development of the malaria parasite. HIV PIs target aspartyl protease [32], and *Plasmodium* species express a number of aspartyl proteases called “plasmepsins.” Ten predicted plasmepsin-encoding genes exist in *P. falciparum* [33]; 4 of them are food vacuole aspartyl proteases, which function in the blood stages for hemoglobin digestion [34]. Previous work has shown that HIV PIs are unlikely to inhibit the food vacuole plasmepsins of *P. falciparum* [35–37]. Plasmepsins are also expressed during the *P. yoelii* preerythrocyte stages [38], so these remain possible targets for the HIV PIs during this stage of the life cycle. Moreover, plasmepsins are present across *Plasmodium* rodent- and human-infecting species [18, 39]. However, whether HIV PIs affect only aspartyl proteases or are less selective and can act against other non–aspartyl protease targets in the malaria parasite remains to be determined. Interestingly, HIV PIs have also been shown to inhibit other apicomplexan parasites, including *Toxoplasma* and *Cryptosporidium* organisms [40].

Drugs that affect preerythrocytic stages are being investigated by a number of groups, given the potential to target the parasite when it is present in lower numbers. Atovaquone and primaquine are the only 2 drugs in practice with known activity against the liver stages. Atovaquone, however, does not act against the preerythrocytic dormant forms of the parasites (hypnozoite forms), which are exhibited in only 2 of the 4 *Plasmodium* species infecting humans—namely, *P. vivax* and *P. ovale* [41]. Atovaquone also can select resistant clones of erythrocytic-stage *P. falciparum*.

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**Table 1. Prolongation of the prepatent period by lopinavir/ritonavir.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average prepatent period, days</th>
<th>Proportion with blood-stage infection</th>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>4.5</td>
<td>6/6</td>
</tr>
<tr>
<td>100 mg/kg QD LPV/r</td>
<td>5.8</td>
<td>6/6</td>
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<tr>
<td><strong>Experiment 2</strong></td>
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<td>4/5</td>
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<tr>
<td>100 mg/kg QD LPV/r</td>
<td>5.0</td>
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**NOTE.** Mice were administered 100 mg/kg lopinavir with 50 mg/kg ritonavir once a day (100 mg/kg QD LPV/r) by gavage. Mice were inoculated with sporozoites intravenously on day 1, and blood smears were checked on days 3–10 after inoculation. The average prepatent period is calculated only from mice that had blood-stage infection. Results from 2 separate experiments are shown.
falciparum [42]. Primaquine does target the dormant forms of P. vivax and P. ovale but cannot be used in pregnant women or those with glucose-6-phosphate dehydrogenase deficiency. Other compounds have been experimentally shown to have an effect against the preerythrocytic stages of Plasmodium species [30, 43–48], but none are currently recommended for human malaria. With few exceptions, the World Health Organization recommends nevirapine-based regimens as first-line therapy and PI-based regimens as second-line therapy for HIV-infected patients in resource-poor settings [49]. If lopinavir/ritonavir regimens are shown to possess antimalarial activity in clinical settings, such knowledge may guide recommendations for HIV therapy in areas in which malaria is endemic. Initiation of lopinavir/ritonavir in young HIV-infected patients may offer secondary prophylactic benefits for modifying malarial infection.

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