Antimalarial asexual stage-specific and gametocytocidal activity of HIV protease inhibitors

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Running Title: Stage-specific activity of HIV protease inhibitors
Abstract

The stage-specific antimalarial activity of a panel of antiretroviral protease inhibitors (PIs), including two non-peptidic PIs (tipranavir and darunavir), was tested in vitro against *P. falciparum*. While darunavir demonstrated limited antimalarial activity (EC$_{50}$>50µM), tipranavir was active at clinically relevant concentrations (EC$_{50}$ 12-21µM). Saquinavir, lopinavir and tipranavir preferentially inhibited the growth of mature asexual stage parasites (24h post invasion). While all of the PIs tested inhibited gametocytogenesis, tipranavir was the only one to exhibit gametocytocidal activity.

Keywords: malaria, *P. falciparum*, antiretroviral protease inhibitors, stage-specific antimalarial activity, gametocytes.
The global distribution of HIV and malaria overlap in many regions of the world (reviewed in (17)). Although data on the number of individuals with both diseases is unavailable, rates of co-infection are likely to be high (7). Furthermore, co-infection often leads to severe disease (4, 12, 19, 20). While the effects of antiretroviral therapy on the outcome of malaria infection are not understood, defining these interactions is important (11, 13, 16, 18). Understanding the antimalarial activity of the antiretroviral protease inhibitors (PIs) (reviewed in (17)) for example, may lead to treatment recommendations that improve clinical outcomes and may also result in the identification of a new antimalarial drug target.

Current data suggest that PIs kill malaria parasites by inhibiting one or more of the six non-digestive vacuole plasmepsins (reviewed in (17)). In the present study we have investigated the stage-specific effects of the PIs on asexual and sexual stage \textit{P. falciparum} parasites in order to help define the antimalarial target/s of these drugs and to help guide partner drug choices in the field. To gain additional structure-activity data and information that may be relevant to co-infected individuals we also examined the activity of the non-peptidic PIs tipranavir (Aptivus®) and darunavir (Prezista®), new generation PIs that are active against HIV-1 strains resistant to first generation PIs (9).

The antimalarial activities of saquinavir, lopinavir, ritonavir, tipranavir, darunavir and chloroquine (diphosphate salt; Sigma) were determined as described (18). Concentrations required to achieve 10, 50 and 90% growth inhibition (±SE) were determined by nonlinear regression curve fit. Each assay was performed in triplicate on at least 2 separate occasions. Stage-specific growth inhibition assays were performed on synchronized parasite cultures (8) at 0 (ring), 24 (trophozoite) and 36 hours (schizont) post synchronization. Cultures were washed post drug exposure, resuspended in
drug-free media and seeded into tissue culture plates containing 0.5 µCi/well $^3$H-hypoxanthine for 40 hours. Incorporation of $^3$H-hypoxanthine was compared to vehicle controls.

Drug induced effects on gametocytogenesis were examined using Pfs16-GFP parasites (3) as previously described (14). Assays were performed in triplicate on three separate occasions. The anti-gametocyte activity of selective PIs was also determined using Pfs16-GFP parasites (3). In these assays gametocytes were sorted from parasite cultures, seeded into microtitre plates (1000 gametocytes and 5% haematocrit) and exposed to drugs or controls for 48 hours. Hydroethidine was used to assess viability. The number of viable gametocytes in test cultures after treatment was compared to controls and results were analysed by one-way ANOVA. Assays were performed in triplicate on two separate occasions.

Tipranavir was active against all parasite lines tested, including the chloroquine-resistant line Dd2 ($EC_{50}=21\pm 2\mu M$) and three chloroquine-sensitive lines (3D7, $EC_{50}=20\pm 2\mu M$; D10, $EC_{50}=12\pm 2\mu M$ and Pfs16-GFP, $EC_{50}=18\pm 4\mu M$). These $EC_{50}$ values are all below the $C_{\min}-C_{\max}$ range (60-185 µM) for this drug in humans (6). Darunavir also inhibited the growth of $P. falciparum$. However, the $EC_{50}$ (Dd2, $EC_{50}=70\mu M$; data not shown) values for this drug was well above clinically achievable levels ($C_{\min}-C_{\max}=0.7-12.4\mu M$) (5). The $EC_{50}$ values of saquinavir (D10 $EC_{50}=3\pm 1\mu M$; 3D7 $EC_{50}=3\pm 3\mu M$; Pfs16-GFP $EC_{50}=5\pm 3\mu M$), lopinavir (D10 $EC_{50}=2\pm 1\mu M$; 3D7 $EC_{50}=2\pm 3\mu M$; Pfs16-GFP $EC_{50}=3\pm 1\mu M$), ritonavir (D10, $EC_{50}=3\pm 1\mu M$; 3D7, $EC_{50}=3\pm 1\mu M$; & Pfs16-GFP, $EC_{50}=5\pm 1\mu M$) and chloroquine (D10, $EC_{50}=23\pm 1nM$; 3D7, $EC_{50}=25\pm 6nM$; Pfs16-GFP, $EC_{50}=23\pm 3nM$) were comparable between parasite lines and similar to previously published values for 3D7 (1, 2).
Saquinavir, lopinavir and tipranavir demonstrated significantly greater growth inhibition (p<0.05) against trophozoite and schizont stages in comparison to ring stages (Figure 1). Similar results were obtained for the drug-resistant Dd2 \textit{P. falciparum} line (data not shown). Chloroquine was used as a control and, as expected, trophozoite stages were more sensitive to this drug than either ring or schizont stages (Figure 1). Each of the four PIs tested also reduced the number of gametocytes produced \textit{in vitro} (Figure 2A). The reduction in gametocytes was dose dependent and statistically significant (P<0.01) when cultures were exposed to EC$_{90}$ levels of ritonavir and all concentrations of tipranavir (Figure 2A). Tipranavir was also able to directly kill gametocytes (Figure 2B; P<0.01).

While saquinavir, ritonavir and lopinavir reduced the numbers of live gametocytes in a dose-dependent fashion, these data did not reach statistical significance (Figure 2B; P>0.05).

Evidence suggesting that PIs may be beneficial to HIV/malaria parasite co-infected individuals is mounting. In addition to possessing antiretroviral activity these drugs also inhibit the growth of malaria parasites (1, 13, 15, 18). In the present study we have extended these data by demonstrating that tipranavir can inhibit the growth of malaria parasites at clinically relevant concentrations (6). Although additional studies including those examining pharmacokinetic drug interactions and the effects of increased plasma proteins on the activity of tipranavir are needed (6), these data further indicate that PIs are likely to be beneficial during HIV/malaria co-infection. The anti-gametocyte activity demonstrated by tipranavir adds an additional dimension to this observation, suggesting that this drug may also have an impact on malaria transmission. These data are novel in that very few antimalarial drugs have anti-gametocyte activity. Indeed most induce gametocytogenesis \textit{in vitro} (14) and as a result can perpetuate transmission and the spread of drug resistant parasites. The observation that none of the PIs induced gametocytogenesis \textit{in vitro} is significant given recent malaria eradication goals and the need for tools to achieve this (10).
To gain an understanding of how HIV PIs kill *P. falciparum* we investigated their effects on individual stages of asexual development. Data from these studies indicate that trophozoite and schizont stages are significantly more sensitive to PIs than ring stage parasites (Figure 1). Taken together with gametocyte inhibition data these results suggest that the primary target of the PIs is likely to be expressed in both gametocytes and intra-erythrocytic parasites. Although expression data (plasmodb.org) requires confirmation and the possibility that the PIs might target different proteases in the different parasite stages cannot be ruled out, plasmepsins V, IX and X appear to be the best candidate targets of these drugs. Future studies investigating these enzymes may identify the antimalarial target of the PIs and help explain the poor antimalarial activity of darunavir. Interestingly, darunavir has a similar structure to amprenavir, another PI with weak antimalarial activity (5, 18).
Figure 1

- **Tipranavir**
- **Saquinavir**
- **Lopinavir**
- **Chloroquine**

Percentage Growth Inhibition vs. Exposure (h)
Figure 2
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References


Figure 1: *In vitro* stage-specific activities of PIs against *P. falciparum* asexual parasites.

Erythrocytes infected with *P. falciparum* line D10 at ring (●), trophozoite (○), or schizont stages (■) were exposed to saquinavir (SQV; 40µM), tipranavir (TPV; 150 µM), lopinavir (LPV; 20µM), and chloroquine (CHQ; 50nM) for 1, 2, 4, 6, or 8 hrs, as described. Data are presented as percentage growth inhibition (+ SE) compared to vehicle controls (taken as 100% growth). Each assay was repeated twice in triplicate.

Figure 2: The activity of selected PIs in gametocyte and gametocyte induction inhibition assays. A) *In vitro* anti-gametocytogenesis activities of selected PIs as determined using transgenic Pfs16-GFP *P. falciparum* parasites and B) the activity of PIs against Pfs16-GFP *P. falciparum* gametocytes. All drugs were assessed at their EC$_{10}$, EC$_{50}$ and EC$_{90}$ values as determined by $^{3}$H-hypoxanthine incorporation against asexually replicating parasites. Bars with * indicate significant changes when compared to vehicle control wells (p<0.01). Pfs16-GFP gametocytes, unlike Pfs16-GFP asexual stage parasites express GFP-tagged Pfs16 (3).