The Effect of Antiretrovirals on *Plasmodium falciparum* Liver Stages

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Abstract

HIV and malaria overlap geographically, but the full impact of different antiretrovirals (ARVs) on malaria remains poorly understood. We examined the antimalarial activity of the HIV protease inhibitors (PI) lopinavir and saquinavir and the non-nucleoside reverse transcriptase inhibitor (NNRTIs) nevirapine on Plasmodium falciparum liver stages. Our results demonstrate that the HIV PI lopinavir inhibits liver stage parasites at clinically relevant concentrations, that is, at drug levels achieved in HIV-infected patients on standard dosing regimens. Because drugs that inhibit liver stages target parasites when they are present in lower numbers, these results might have implications for eradication efforts.

Keywords

HIV; malaria; antiretroviral therapy; liver stages; eradication

Introduction

HIV and malaria co-occur in many parts of the world, and evidence suggests that HIV and *Plasmodium falciparum* malaria, both commonly found in sub-Saharan Africa in epidemic proportion, exert co-pathogenic effects [1].

Previous work shows HIV protease inhibitors (PIs), but not non-nucleoside reverse transcriptase inhibitors (NNRTIs), kill *P. falciparum* asexual blood stages [2], and clinical

studies are beginning to examine the antimalarial effects of PIs vs. NNRTIs in the context of antiretroviral therapy (ARV) on malaria [3, 4]. We have previously shown in rodent models of malaria that HIV PIs also appear to have more potent effect against *Plasmodium* compared to NNRTIs [5, 6]. However, antiretroviral effect on *P.falciparum* liver stages has not been reported. Currently, the World Health Organization (WHO) recommends HIV management with combination antiretroviral therapy (ARV), with first line regimens including an NNRTI (in children, nevirapine) and 2 nucleoside reverse transcriptase inhibitors (NRTIs) with few exceptions, and second line regimens including an HIV PI (in children, lopinavir-ritonavir) and 2 NRTIs [7, 8]. Since young children are at highest risk for severe malaria, we set out to investigate the effects of the HIV PI lopinavir and the NNRTI nevirapine on liver stage *Plasmodium falciparum*. Saquinavir was also investigated given prior results indicated it was comparably potent *in vitro* against liver stage rodent malaria [5]. Because these drugs are used in HIV-infected patients in malaria-endemic areas, and because there is evidence that indicates PIs may affect liver stage *Plasmodium* differently than NNRTIs [5, 6], the effects of various ARV components on *Plasmodium falciparum* requires further investigation.

Malaria infection begins with the bite of an infected female Anopheles mosquito, releasing sporozoites, the infective form of the parasite. Sporozoites travel to the liver and invade hepatocytes, developing into liver stages, or exoerythrocytic forms (EEFs). During these events, the host is asymptomatic, and parasite numbers are low [9]. Infected hepatocytes release merozoites, which invade erythrocytes, initiating the phase of infection responsible for all clinical symptoms of malaria. Most antimalarials target this symptomatic asexual blood stage. However, preventing malaria blood stage infection with drugs targeting liver stages might impact transmission and might contribute to malaria eradication efforts. Here, we describe our investigations of ARV effects on liver stages of *P. falciparum* malaria parasites at and above clinically relevant concentrations, or drug levels achieved in HIV-treated patients on standard dosing regimens [2, 5, 6, 10–12] (for a summary list of clinically relevant concentrations, please see reference [2]).

Methods

Lopinavir, saquinavir, and nevirapine were tested. Drugs were obtained from the NIH AIDS Research and Reference Reagent Program. Stocks were diluted to 5 mM with dimethyl sulfoxide (DMSO), and were tested at a range at and above clinically relevant concentrations [2, 5, 6, 10–12]. *In vitro P. falciparum* liver stage development assays were performed as previously described, with few modifications [13, 14]. Briefly, *P. falciparum* sporozoites were isolated from mosquitoes day 14 after the infective blood meal using the Ozaki method [15]. Then, 7.5×10^4 sporozoites per well were seeded onto monolayers of HC04 cells grown in standard 8-well Nunc ® Lab-Tek® chamber slides (well surface area, 0.8 cm^2) and incubated in the presence of lopinavir, saquinavir, or nevirapine prepared in DMSO in DMEM culture medium, with media and drug and replenished daily. Control wells had culture medium only or DMSO and culture medium. On day 4, EEFs were fixed and stained with a monoclonal antibody specific for *Plasmodium* HSP70 [14, 16], followed by a fluorophore-conjugated secondary antibody. Samples were mounted with Vecta Shield (Vector Laboratories, Burlingame, CA) with DAPI nuclear stain (Sigma-Aldrich, St. Louis,

MO) EEFs were counted with an epifluorescence microscope (model Olympus BX50 or BX51). Each developing EEF visualized was counted. Experiments were run in duplicates per condition, and 2 independent experiments were performed. Statistics were performed using Kruskal-Wallis with Dunn's post-test on combined experiments values to identify statistically significant differences between treated and control. All values were normalized to DMSO control, and no significant differences were detected between DMSO control and infectivity controls. Statistical analyses were performed using GraphPad Prism software (version 5).

Results

The HIV PI lopinavir reduced *P. falciparum* developing EEFs at clinically relevant concentrations that would be achieved with ritonavir boosting ($p<0.001$). Saquinavir and nevirapine also reduced *P. falciparum* developing EEFs ($p<0.01$), but only at concentrations exceeding clinical relevance (Fig.1).

Conclusion/Discussion

These results show that the PI lopinavir has anti-liver stage specific activity at clinically relevant concentrations. Saquinavir and nevirapine demonstrate anti-liver stage activity, but only at concentrations exceeding clinical relevance. The results are consistent with prior data which demonstrates that lopinavir kills rodent malaria liver stage parasites [5], and *Plasmodium falciparum* asexual blood stage [2, 17–20] and transmission stage parasites ([21, 22]) at concentrations that or at or are below clinically relevant concentrations. We, and others have also shown that NNRTIs have little to no effect on malaria parasites in liver [6], asexual blood [2], and transmission stage parasites [22], and any effect that is seen only occurs at concentrations exceeding clinical relevance. HIV PIs were originally tested against *P. falciparum* blood stages as these drugs inhibit the aspartyl protease of HIV [19, 23], and it was hypothesized similar inhibition may occur with *Plasmodium* aspartyl proteases, although this requires further investigation. For the NNRTIs, anti-*Plasmodium* activity observed at concentrations exceeding clinical relevance may come from increased oxidative stress on the parasite [6].

A possible limitation of these experiments may be different drug metabolism within *in vitro* vs. *in vivo* systems. Reassuringly, the human-derived HC04 cell lines used in this study were shown in a series of previous investigations to express a variety of Phase 1 and 2 drug metabolizing enzymes, including those involved in lopinavir [11], saquinavir [12], and nevirapine [10] biotransformation [13, 24]; these primarily include cytochrome P450 3A4 (CYP3A4) (lopinavir, saquinavir, and nevirapine) and CYP2B6 (nevirapine). Thus, the *in vitro* systems should reflect *in vivo* metabolic activity. However, subtle drug metabolism differences may exist. Field studies are required to determine whether PIs like lopinavir offer benefit in reducing malaria burden through an anti-liver stage effect.

Acknowledgements

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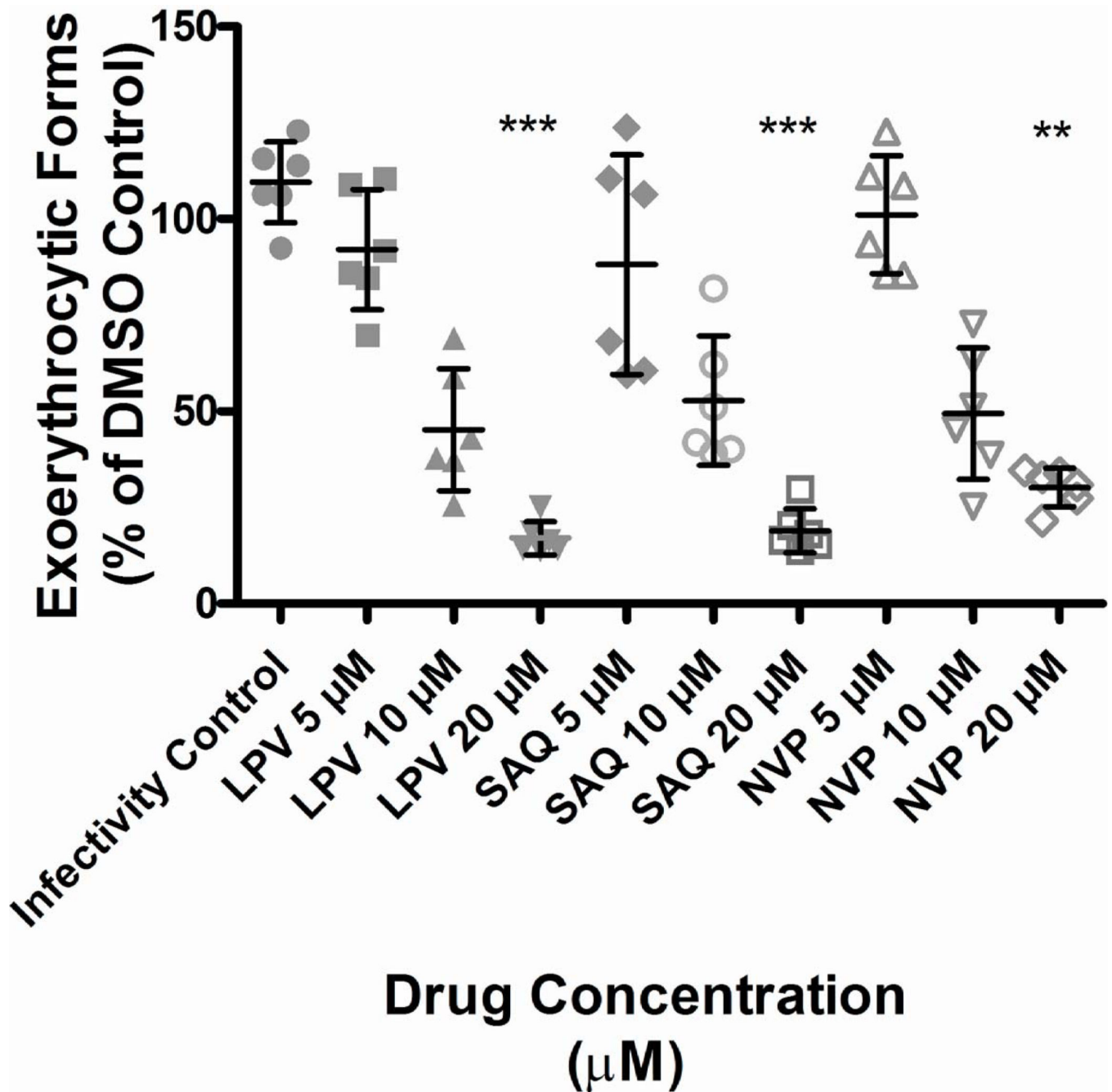
Figure 1. HIV PIs and NNRTIs inhibit *Plasmodium falciparum* liver stage parasites (exoerythrocytic forms/EEFs) at the high end of clinically relevant concentrations

P. falciparum sporozoites were seeded onto monolayers of HC04 cells and incubated in the presence of indicated ARTs (lopinavir, LPV; saquinavir, SAQ; nevirapine, NVP) at the indicated concentrations. On day 4, cells were fixed and stained with a monoclonal antibody specific for *Plasmodium* HSP70 and counted on an epifluorescence microscope. Shown are the combined results from 2 independent experiments, with horizontal lines indicating the mean. Statistics were performed using Kruskal-Wallis with Dunn's post-test on combined experiments values to identify statistically significant differences between treated and

control. All values were normalized to DMSO control and expressed as percent of DMSO control. No significant differences were detected between DMSO control and infectivity controls (cell medium only). Three stars indicate $p < .001$, two stars indicate $p < 0.01$. For EEF number, significant differences were found between treated and control groups at the highest doses only for each ART tested (lopinavir and saquinavir each compared to control, $p < 0.001$; nevirapine, $p < 0.01$). Only lopinavir significantly inhibited EEF development at clinically relevant concentrations.