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Altered *Plasmodium falciparum* Sensitivity to the Antiretroviral Protease Inhibitor Lopinavir Associated with Polymorphisms in *pfmdr1*

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ABSTRACT The HIV protease inhibitor lopinavir inhibits *Plasmodium falciparum* aspartic proteases (plasmepsins) and parasite development, and children receiving lopinavir-ritonavir experienced fewer episodes of malaria than those receiving other antiretroviral regimens. Resistance to lopinavir was selected *in vitro* over ~9 months, with ~4-fold decreased sensitivity. Whole-genome sequencing of resistant parasites showed a mutation and increased copy number in *pfmdr1* and a mutation in a protein of unknown function, but no polymorphisms in plasmepsin genes.

KEYWORDS malaria, *Plasmodium falciparum*, drug sensitivity, drug resistance, *pfmdr1*, aspartic protease, antiretroviral, lopinavir, HIV, drug resistance mechanisms

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Infection with *Plasmodium falciparum*, the most virulent human malaria parasite, causes hundreds of millions of illnesses and hundreds of thousands of deaths each year (1). Despite recent progress in some regions, the treatment and control of the disease are challenged by increasing resistance to available therapies (2). New drugs to treat malaria are needed. One approach is to repurpose drugs now used for other indications to treat or prevent malaria.

The *P. falciparum* genome sequence predicts the presence of 10 aspartic proteases, known as plasmepsins (3). Plasmepsins I, II, III (also known as histo-aspartic protease), and IV hydrolyze hemoglobin in the plasmodial food vacuole, in concert with other proteases, to provide amino acids for erythrocytic parasites (4). Plasmepsin V is an endoplasmic reticulum protease that cleaves proteins bound for export to the erythrocyte (5, 6). The functions of plasmepsins VI to X are unknown, with different enzymes believed to be active in erythrocyte- and mosquito-stage parasites (7, 8).

The HIV protease is also an aspartic protease (9), and inhibitors of this enzyme are among our most important antiretroviral drugs (10). A number of antiretroviral protease inhibitors have been shown to inhibit plasmepsins (11), to be active against cultured malaria parasites (11, 12), and to effectively treat murine malaria (13). Lopinavir, which is used to treat HIV in combination with ritonavir, is active against *P. falciparum* at low micromolar concentrations that are below the levels achieved by standard dosing (11). HIV-infected Ugandan children who received lopinavir/ritonavir had decreased incidence of malaria compared to those receiving a regimen that did not include a protease inhibitor (14). The impact of lopinavir/ritonavir appeared to be mediated principally by prolonged exposure to the antimalarial lumefantrine after therapy, due to inhibition of metabolism by ritonavir, rather than by protease inhibition, as the effect was greatest in episodes following prior therapy with artemether-lumefantrine. However, considering only the first episodes of malaria, and thus remov-

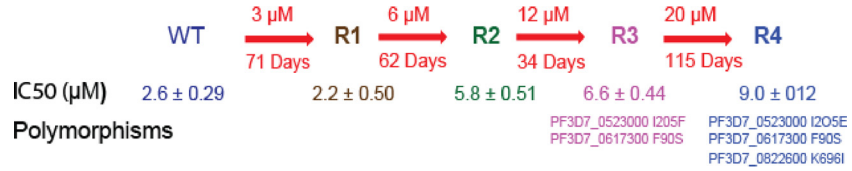

FIG 1 Selection of *P. falciparum* with decreased sensitivity to lopinavir. Each selection from wild type (WT) to generations R1 to R4 is indicated by an arrow, with the selection concentration and time indicated. Sensitivities of selected strains are shown (50% inhibitory concentration [IC_{50}]; mean of triplicate measures ± standard error of the mean [SEM]). WT sensitivity is the mean of assessments at each time point for cultures grown in parallel without lopinavir. Polymorphisms in R3 and R4 parasites are shown. The copy numbers of PFE1150w were 1 in WT and 4 in R3 and R4 parasites.

ing the influence of prior antimalarial therapy, there was a trend toward decreased malaria in the lopinavir-ritonavir-treated group, suggesting a direct impact of inhibition of aspartic proteases by lopinavir on malarial incidence. Other studies in women from 7 African countries with varied malaria risk did not show a decrease in malaria in those treated with lopinavir-ritonavir compared to other antiretroviral regimens (15, 16). In any event, as aspartic protease inhibitors may have promise as new antimalarial agents (17, 18), and as protease inhibitors now used to treat HIV infection protect against malaria in some settings, we were interested in characterizing the ease of selection of resistance to these agents and parasite alterations associated with decreased drug sensitivity. We therefore selected for decreased sensitivity to lopinavir and characterized selected parasites.

Selection of lopinavir-resistant malaria parasites. W2 strain *P. falciparum* was obtained from the Malaria Research and Reference Reagent Resource Center (https://www.beiresources.org/MR4Home.aspx) and cultured at 2% hematocrit in RPMI 1640 (Invitrogen) medium supplemented with 0.5% AlbuMAX II (GIBCO Life Technologies), 2 mM L-glutamine, 100 mM hypoxanthine, 5 g/ml gentamicin, 28 mM $NaHCO_3$, and 25 mM HEPES at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2, with three parallel cultures of 6×10^7 parasites subjected to stepwise increasing concentrations of lopinavir (Fig. 1). Lopinavir was obtained from the NIH AIDS Reagent Program (https://www.aidsreagent.org/). At each step of selection, parasites were initially undetectable on Giemsa-stained smears followed by regrowth, suggesting selection of mutations allowing growth under drug pressure. After each step of selection, we assessed parasite sensitivity by counting fluorescently stained parasites incubated with serial dilutions of lopinavir, as previously reported for other compounds (19), and we cloned parasites by limiting dilution. We then characterized wild-type and resistant parasites by whole-genome sequencing. Of note, altered sensitivity was selected slowly, with only incremental changes in parasite sensitivity, and about 9 months of incubation with lopinavir was required to select for parasites with an ~4-fold decrease in lopinavir sensitivity after 4 cycles of selection.

Sequencing of lopinavir-resistant parasites. Wild-type W2 strain parasites and those with decreased lopinavir sensitivity were cloned by limiting dilution and then characterized by whole-genome sequencing, as reported previously (19). In brief, genomic DNA libraries were prepared, libraries were barcoded with unique sets of indices, fragments of 360 to 560 bp were extracted, the fragments were amplified by limited-cycle PCR, libraries were pooled, and sequencing was performed at the UCSF Center for Advanced Technology on a HiSeq 2000 system (Illumina). Sequence data for each library were aligned with the 3D7 reference genome (PlasmoDB v26) using Bowtie (20), discarding reads with >1 nucleotide mismatch and multiple alignments across the genome. For the identification of single-nucleotide polymorphisms (SNPs), reads were compared to those of the parental strain, and the top 200 SNPs per chromosome were chosen based on the frequency of conflicting nucleotides per position in the genome and filtered (21). SNPs were considered legitimate if they had >10 reads and a frequency of ≥80%. Searches for novel SNPs included only nonsynonymous SNPs in

exons and excluded the hypervariable genes *pfemp1*, *rifin*, and *stevor*. For analysis of copy number, raw reads were filtered for quality using the PriceSeqFilter module (22), reads were aligned to the 3D7 genome with Bowtie2 (20) using the very sensitive Flag, and only unique reads were retained. Copy number was analyzed using a custom script to compare parental, selected, and 3D7 genomes. Reads were normalized for the total number of reads for different strains.

Lopinavir-resistant parasites demonstrated an SNP and altered copy number in *pfmdr1*. Two clones were analyzed from each of the two most advanced generations of resistance selection (R3 and R4 [Fig. 1]). Both clones from both generations had an SNP (A613T, encoding I205F) in the *pfmdr1* gene, which encodes the multidrug resistance P-glycoprotein homologue (PF3D7_0523000) and an SNP (T269C, encoding F90S) in a gene of unknown function on chromosome 6 (PF3D7_0617300). The most resistant R4 clones had an additional SNP (A2087T, encoding K696I) in the PF3D7_0822600 gene, which is predicted to encode a homologue of Sec23, a component of a complex that forms membrane vesicles at the endoplasmic reticulum in other eukaryotes (23). In addition, *pfmdr1* was amplified 4-fold in R3 and R4 resistant parasites. There were no SNPs or increases in copy number observed in any of the 10 plasmepsin genes.

In summary, we selected for resistance in *P. falciparum* to the HIV protease inhibitor lopinavir. Resistance was selected slowly, and only modest decreases in drug sensitivity were seen after months of culture with lopinavir. Surprisingly, resistant parasites did not have alterations in plasmepsin genes, the presumed targets of lopinavir. Rather, they showed an SNP and increased copy number in *pfmdr1*, which encodes a putative transporter on the surface of the *P. falciparum* food vacuole, in which plasmepsins I to IV contribute to the essential process of hemoglobin hydrolysis (4). Alterations in *pfmdr1*, including both SNPs and increased copy number, are associated with altered sensitivity to a number of antimalarials (2). SNPs were also seen in resistant parasites in two other genes. Available data suggest that tested HIV protease inhibitors do not have adequate antimalarial potency for consideration as new antimalarial drugs. However, when used to treat HIV infection, lopinavir/ritonavir decreased the incidence of malaria (14). Consideration of other plasmepsin inhibitors as potential antimalarial agents is ongoing (17, 18). Our results, showing limited resistance selection by lopinavir and a lack of changes in plasmepsin genes in parasites with moderate resistance, offer some confidence that the development of resistance to aspartic protease inhibitors may be slow.

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