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Sunil Parikh
University of California - San Francisco

Jun Liu
Washington University School of Medicine in St. Louis

Puran Sijwali
University of California - San Francisco

Jiri Gut
University of California - San Francisco

Daniel E. Goldberg
Washington University School of Medicine in St. Louis

See next page for additional authors

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NOTES

Antimalarial Effects of Human Immunodeficiency Virus Type 1 Protease Inhibitors Differ from Those of the Aspartic Protease Inhibitor Pepstatin

Sunil Parikh,[1]* Jun Liu,[2] Puran Sijwali,[1] Jiri Gut,[1] Daniel E. Goldberg,[2] and Philip J. Rosenthal[1]

Department of Medicine, San Francisco General Hospital, University of California, San Francisco, California,[1] and Department of Medicine and Department of Molecular Microbiology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri[2]

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Human immunodeficiency virus type 1 protease inhibitors (HIVPIs) and pepstatin are aspartic protease inhibitors with antimalarial activity. In contrast to pepstatin, HIVPIs were not synergistic with a cysteine protease inhibitor or more active against parasites with the cysteine protease falcipain-2 knocked out than against wild-type parasites. As with pepstatin, HIVPIs were equally active against wild-type parasites and against parasites with the food vacuole plasmepsin aspartic proteases knocked out. The antimalarial mechanism of HIVPIs differs from that of pepstatin.

Human immunodeficiency virus type 1 (HIV-1) and malaria are coendemic throughout much of the developing world. HIV-1 expresses an aspartic protease, which is an important drug target (3). In addition to their important antiretroviral activity, HIV-1 protease inhibitors (HIVPIs) are active against *Plasmodium falciparum* in vitro and against rodent malaria parasites in murine models (7, 9, 13). The predicted targets of these inhibitors are plasmepsins, a family of aspartic proteases of malaria parasites. A number of plasmepsins act in concert with falcipain cysteine proteases and other enzymes to hydrolyze hemoglobin in the *P. falciparum* food vacuole (5, 8). Several HIVPIs inhibit the food vacuole protease plasmepsin II (7) and a homologous protease of the rodent parasite *Plasmodium chabaudi* (6). Pepstatin, the most-studied aspartic protease inhibitor, also exhibits activity against cultured malaria parasites and inhibits several plasmepsins (2, 6). As the antimalarial activity of HIVPIs may have important implications in areas where those treated for HIV-1 infection are at risk of malaria, and as both HIVPIs and pepstatin may serve as leads for new antimalarial agents, it was of interest to compare their antimalarial mechanisms of action.

Insight into the antimalarial mechanisms of protease inhibitors came from studies that showed that cysteine protease inhibitors [*N*-(*trans*-epoxysuccinyl)-L-leucine-4-guanidinobutylamide (E-64)] and aspartic protease inhibitors (pepstatin) display marked synergy against malaria parasites (1, 10). Further supporting an important interaction between these two classes of proteases, pepstatin had markedly enhanced activity against *P. falciparum* parasites in which the gene for the cysteine protease falcipain-2 was disrupted (11). It was of interest to determine if HIVPIs had effects similar to those of pepstatin.

We evaluated the HIVPI lopinavir for synergy with E-64. *P. falciparum* (W2 strain) parasites were cultured in RPMI medium supplemented with 10% serum and synchronized with 5% D-sorbitol as previously described (11). Ring stage parasites were incubated with study drugs (0.039 to 10 μM, from stock solutions concentrated 1,000-fold in dimethyl sulfoxide [DMSO]) or with equivalent concentrations of DMSO for 48 h, fixed with 1% formaldehyde in phosphate-buffered saline for 48 h, and labeled with 1 nM YOYO-1 dye (Molecular Probes) in 0.1% Triton X-100 in phosphate-buffered saline. Parasitemias were determined from dot plots acquired with a FACSort flow cytometer, and 50% inhibitory concentration (IC_{50}) values were calculated as previously described (11, 12). Potential synergy was evaluated as the sum of the fractional inhibitory concentrations (sum FIC) by the following equation: sum FIC = [(IC_{50} drug A in combination)/(IC_{50} drug A alone)] + [(IC_{50} drug B in combination)/(IC_{50} drug B alone)]. The sum FIC value for lopinavir and E-64 was 2.04 ± 0.48 (mean ± standard deviation of results from two experiments, each done in triplicate). Thus, lopinavir and E-64 (Sigma-Aldrich) showed no evidence of synergism, but rather borderline antagonism. In contrast, E-64 and pepstatin have shown marked synergy with a sum FIC value of 0.54 ± 0.16 (10).

To further characterize the antimalarial mechanism of HIVPIs, we tested the compounds against *P. falciparum* parasites with disrupted food vacuole proteases. For plasmepsin knockout parasites, previously described 3D7 strain parasites were used (5). For falcipain-2 knockout parasites, procedures very similar to those previously described were used (11). Briefly, 3D7 strain parasites were transfected with the pHTK-ΔFP2 plasmid, selected with WR99210 until integration of the plasmids was detected, enriched for recombinant parasites through negative selection with ganciclovir, and cloned to obtain pure recombinant parasites. Wild-type 3D7 and plasmepsin knockout parasites were incubated in microwell cultures in the presence of serial dilutions of lopinavir, ritonavir, and saquinavir (0.025 to 150 μM, from 1,000-fold-concentrated

* Corresponding author. Mailing address: University of California San Francisco, Box 0811, San Francisco, CA 94110. Phone: (415) 206-8687. Fax: (415) 648-8425. E-mail: sparikh@medsfgh.ucsf.edu.

TABLE 1. Activity of HIV-1 protease inhibitors against *P. falciparum* plasmepsin knockout parasites

Drug	IC_{50} (μM) for *P. falciparum*[a]				
	Wild type	PMPI KO	PMPII KO	HAP KO	PMPI/IV KO
Saquinavir	12.2 ± 0.3	11.6 ± 0.6	11.1 ± 0.3	15.1 ± 2.7	13.4 ± 0.7
Ritonavir	12.2 ± 0.4	11.4 ± 1.0	11.7 ± 0.4	14.5 ± 0.5	12.4 ± 0.6
Lopinavir	3.0 ± 0.4	2.4 ± 0.2	2.5 ± 0.1	3.2 ± 0.2	3.9 ± 0.7

[a] IC_{50} data are means ± standard deviations of results from three experiments. Abbreviations: PMP, plasmepsin; KO, knockout; HAP, histoaspartic protease.

TABLE 2. Activity of HIV-1 protease inhibitors against *P. falciparum* falcipain-2 knockout parasites

Drug	IC_{50} (μM) for *P. falciparum*[a]		Knockout IC_{50} (% of wild-type IC_{50})
	Wild type	Falcipain-2 knockout	
Saquinavir	5.7 ± 0.8	7.6 ± 1.7	133
Ritonavir	7.9 ± 1.1	8.9 ± 0.9	113
Indinavir	25.2 ± 4.4	26.6	105
Nelfinavir	21.8 ± 2.4	24.7	113
Amprenavir	71.24 ± 16.1	84.7	118
Lopinavir	2.5 ± 0.4	3.4 ± 0.8	138
Atazanavir	35.0 ± 7.4	42.0	120
Pepstatin	7.5 ± 0.4	0.04 ± 0.01	0.5
E-64	2.4 ± 0.4	1.4 ± 0.1	58

[a] IC_{50} data are means ± standard deviations of results from three experiments.

stocks in DMSO) or with equivalent concentrations of DMSO for 44 h, beginning at the ring stage, and then 0.5 μCi of [^{3}H]hypoxanthine (178.7 Ci/mmol; Perkin Elmer) was added. The incubation was continued for 16 h, the parasites were harvested, the hypoxanthine uptake rates of treated and control parasites were compared, and IC_{50} values were generated as previously described (5). The antimalarial activities of seven HIVPIs against 3D7 wild-type and falcipain-2 knockout parasites were evaluated by assessing the fluorescence of YOYO-1-stained parasites and determining IC_{50} values using fluorescence-activated cell sorter-based analysis as described above (11, 12).

HIVPIs had similar activities against control, plasmepsin knockout (Table 1), and falcipain-2 knockout (Table 2) parasites. Discrepancies between reported IC_{50} values in Tables 1 and 2 likely reflect differences between the [^{3}H]hypoxanthine uptake and fluorescence-activated cell sorter-based assay methods. Considering the actions of other protease inhibitors, E-64 was about twice as active against falcipain-2 knockout and plasmepsin knockout parasites as it was against control parasites, as previously described (5). Pepstatin (Sigma-Aldrich) was about equally active against control and plasmepsin knockout parasites but was much more active against falcipain-2 knockout parasites, all consistent with prior findings (5, 11).

Our results identify major differences between the antimalarial activities of pepstatin and HIVPIs. Pepstatin, the most widely available broadly active aspartic protease inhibitor (3), inhibits multiple plasmepsins and rapidly kills cultured parasites, but its antimalarial mechanism of action is uncertain. For both pepstatin and HIVPIs, activities were similar against wild-type and plasmepsin knockout parasites. These results suggest the functional redundancy of plasmepsins but do not shed light on the mechanism of HIVPIs. In contrast, the activities of pepstatin and HIVPIs differed markedly when evaluated for antimalarial synergy with the cysteine protease inhibitor E-64 or for activity against falcipain-2 knockout parasites. Pepstatin shows strong synergy with cysteine protease inhibitors (10), and it is remarkably more potent against falcipain-2 knockout parasites than against wild-type parasites (11). These results suggest important biological interactions between falcipains and plasmepsins. Surprisingly, the HIVPIs were not synergistic with E-64 and were not more active against falcipain-2 knockout parasites than against wild-type parasites. Thus, although we cannot yet identify specific biological targets for either pepstatin or HIVPIs in malaria parasites, our results strongly suggest that the compounds act differently.

P. falciparum encodes 10 predicted aspartic protease genes (14). In addition to the four food vacuole plasmepsins (I to IV), another quite different aspartic protease, plasmepsin V, was recently characterized (4). This protease is not located in the food vacuole and does not bind pepstatin. The HIV-1 protease is also quite different from the food vacuole plasmepsins (15). These structural differences may offer insight into the differential effects of the HIVPIs and pepstatin. Previously we showed that HIVPIs inhibit recombinant plasmepsin II (7), but the intracellular target of these inhibitors is unknown. Thus, pepstatin may target food vacuole plasmepsins, which are dependent on food vacuole cysteine proteases for maximal activity, while HIVPIs may target other aspartic proteases, such as plasmepsin V. In addition to differential inhibition of parasite proteases, various effects may be due to differential access to the food vacuole or other cellular compartments. Alternatively, either pepstatin or HIVPIs might exert antimalarial effects that are unrelated to protease inhibition. Our results do not yet identify a specific mechanism of action for HIVPIs, but they offer the surprising finding that HIVPIs do not, as would have been predicted, act in the same manner as pepstatin. Further research into the precise mode of action of HIVPIs and other antimalarial aspartic protease inhibitors is warranted to provide insight into the development of protease inhibitors as new antimalarial drugs and to understand the means by which antiretroviral drugs may offer protection against malaria.

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