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Comparison of Efficacies of Cysteine Protease Inhibitors against Five Strains of *Plasmodium falciparum*

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Falcipain-2, a cysteine protease and essential hemoglobinase of *Plasmodium falciparum*, is a potential antimalarial drug target. We compared the falcipain-2 sequences and sensitivities to cysteine protease inhibitors of five parasite strains that differ markedly in sensitivity to established antimalarial drugs. The sequence of falcipain-2 was highly conserved, and the sensitivities of all of the strains to falcipain-2 inhibitors were very similar. Thus, cross-resistance between cysteine protease inhibitors and other antimalarial agents is not expected in parasites that are now circulating and falcipain-2 remains a promising chemotherapeutic target.

Malaria remains a major problem throughout developing parts of the world (7). Complicating an already difficult situation, malaria parasites, especially *Plasmodium falciparum*, are increasingly resistant to available drugs (15). New antimalarial agents are thus urgently needed. Among potential new targets for chemotherapy is the *P. falciparum* cysteine protease falcipain-2. This protease is a principal cysteine protease and essential hemoglobinase of erythrocytic *P. falciparum* trophozoites (11, 14). In in vitro studies, inhibitors of falcipain-2 blocked parasite hemoglobin hydrolysis and halted the development of cultured parasites (12, 13). In addition, falcipain-2 inhibitors cured mice of otherwise lethal malaria infections (8, 10). Falcipain-2 inhibitors are therefore worthy of additional evaluation as potential new antimalarial drugs.

An important consideration in antimalarial drug discovery is the ability of new agents to treat all strains of *P. falciparum* that may infect human populations. The capacity of falcipain-2 inhibitors to block the development of different strains of *P. falciparum* has not previously been evaluated in detail. In addition, it is unclear whether cross-resistance occurs between established antimalarials and falcipain-2 inhibitors. As resistance to chloroquine and other agents may be mediated, at least in part, by mutations in proteins that direct the transport of antimalarials, it remains plausible that mutations that cause resistance to chloroquine may lead to resistance to unrelated compounds, including falcipain-2 inhibitors (5, 15). Indeed, although mechanisms of resistance to many drugs remain uncertain, *P. falciparum* strains commonly exhibit resistance to multiple drugs with diverse mechanisms of action. Some strains might also exhibit resistance to cysteine protease inhibitors due to differences in the falcipain-2 drug target. We have therefore compared the sequences of falcipain-2 and sensitivities to falcipain-2 inhibitors of five strains of *P. falciparum* that vary greatly in sensitivity to established antimalarial drugs.

Five well-characterized laboratory strains of *P. falciparum* were cultured and synchronized in medium containing 10% human serum as previously described (12) (Table 1). The strains were originally provided by Dennis Kyle, Walter Reed Army Institute of Research (W2 and D6); James Leech, University of California, San Francisco (ItG); and the Malaria Research and Reference Reagent Resource Center (HB3 and Dd2). DNA was purified from schizont-stage parasites by phenol extraction and isopropanol precipitation as previously described (14). The falcipain-2 gene was amplified with *Taq* DNA polymerase (GIBCO-BRL) from the genomic DNA of each strain using forward (5′GTGTATTTTATTTTGTAGCAAGAACGTTTTGTG3′) and reverse (5′TGACAAGCTTATTCAATTAATGGAATGAATGCATCAGTACC3′) primers that spanned the gene. PCR products were gel purified using the QIAquick gel extraction kit (Qiagen) and ligated into the pCR2.1-TOPO vector. TOP10 *Escherichia coli* was transformed with the vectors, and plasmid DNA was purified and sequenced in both directions by dideoxy sequencing. Any potential sequence polymorphisms based on comparisons with the known sequence were confirmed by repeat sequencing of additional clones. For Southern analysis, 10 μg of genomic DNA from each strain was digested with restriction endonucleases, electrophoresed on a 0.7% agarose gel, and transferred onto a nylon membrane (Amersham). The membrane was hybridized overnight with an α-^{32}P-labeled probe (multiprime DNA labeling system [Amersham]) that encoded the most carboxy-terminal 35 amino acids of the prodomain and the complete mature domain of falcipain-2 and then washed under high-stringency conditions as previously described (14).

The antimalarial effects of cysteine protease inhibitors were assessed with three assays, which measured effects on parasite hemoglobin degradation, development, and metabolism, as discussed below. The protease inhibitors were morpholine urea-phenylalanine-homophenylalanine-fluoromethyl ketone (a gift from Robert Smith, Prototek, Inc.) and the vinyl sulfones morpholine urea-leucine-homophenylalanine-phenyl vinyl sulfone, *N*-methyl piperazine urea-leucine-homophenylalanine-phenyl vinyl sulfone, and *N*-methyl piperazine urea-leucine-homophenylalanine-naphthalene vinyl sulfone (all gifts from James Palmer, Axys, Inc.). Chloroquine phosphate was from Sigma. Ring-stage parasites were incubated with 10-fold serial dilutions of inhibitors (prepared from 1,000× stocks in dimethyl

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TABLE 1. Features of the five *P. falciparum* strains used in this study

Strain	Origin	Sensitivity[a]					
		CQ	Qui	Pyr	Cyc	Sul	MQ
D6	Sierra Leone	S	S	S	S	S	S/R
Dd2	Indochina	R	R	R	—	—	R
HB3	Honduras	S	R	R	S	S	S
ItG	Brazil	R	—	S	—	—	—
W2	Indochina	R	R	R	R	R	S

[a] The sensitivity data shown is based on other reports (3, 4, 6, 9, 16). The drugs studied were chloroquine (CQ), quinine (Qui), pyrimethamine (Pyr), cycloguanil (Cyc), sulfadoxine (Sul), and mefloquine (MQ). Results are reported as sensitive (S), resistant (R), intermediate (S/R), or unknown (—). Some results have varied between laboratories.

sulfoxide) in microwell cultures. To evaluate hemoglobin degradation, smears were prepared after 24 h of growth and the morphologies of treated and control (0.1% dimethyl sulfoxide) parasites were inspected after staining with Giemsa. A block in hemoglobin degradation was manifested as the appearance of swollen, dark-staining food vacuoles in the majority of parasites (11). Parasite development was assessed after 48-h incubations by comparing ring-stage parasitemias in treated and control cultures (12). Parasite metabolism was assessed in a modification of a standard assay (2) by incubating ring-stage cultures with inhibitors and [^{3}H]hypoxanthine (1.2 μCi per well; Dupont-NEN) for 18 h and comparing the levels of [^{3}H]hypoxanthine uptake by treated and control cultures as previously described (12). For assays of development and metabolism, all values were normalized to percent control activity and 50% inhibitory concentrations (IC_{50}s) were calculated using the Prism 3.0 program (GraphPad Software) with data fitted by nonlinear regression to the variable-slope sigmoidal dose-response formula $y = 100/[1 + 10^{(\log IC_{50} - x)H}]$, where H is the Hill coefficient (slope factor). Goodness of fit was documented by R^2 values of 0.93 to 0.99 for all of the curves generated.

The predicted amino acid sequences of the five falcipain-2 genes (GenBank accession numbers AF282975 to AF282979) were identical, except for two conservative substitutions, Val_{51} to Ile_{51} in a hydrophobic putative transmembrane domain of the ItG sequence and Gln_{414} to Glu_{414} in both ItG and HB3 at a site that is near an active-site His but is poorly conserved among cysteine proteases of *P. falciparum* (14) and other organisms (1). Thus, the sequences of falcipain-2 were identical among *P. falciparum* strains with origins on three different continents, except for two conservative substitutions between the sequences of New World and Old World isolates. By Southern analysis, hybridization patterns for DNA reacted with *Bgl*II, *Eco*RV, and *Xmn*I and probed with the falcipain-2 gene were identical for all of the five strains (data not shown). This result indicates the presence of two closely related cysteine protease genes, presumably encoding falcipain-2 and falcipain-3 (14), and suggests that the genetic organization of falcipain-2 and related genes is the same for each isolate. Our results suggest that variations in the enzyme target will not complicate drug discovery efforts directed against falcipain-2.

The cysteine protease inhibitors described in this report have previously been shown to strongly inhibit falcipain-2 and demonstrate marked antimalarial effects (8, 10, 12). In our studies of five *P. falciparum* strains, all inhibitors strongly blocked hemoglobin degradation at a 1 nM concentration. Parasite development was inhibited at subnanomolar concentrations, and metabolism was blocked at low nanomolar concentrations by all four of the compounds (Table 2). Importantly, results were very consistent among the five strains studied and small differences identified between the strains appeared to be randomly distributed, without any correlation between the activity of cysteine protease inhibitors and sensitivity to antimalarial drugs.

Our results suggest that cross-resistance between cysteine protease inhibitors and other commonly used antimalarials is not a significant concern. The four peptidyl inhibitors studied were very potent and equally effective against five *P. falciparum* strains that differed greatly in sensitivity to chloroquine and other antimalarials. Similar results have also been obtained with nonpeptidyl falcipain-2 inhibitors (A. Singh and P. J. Rosenthal, unpublished data). These results suggest that it is unlikely that any *P. falciparum* parasites currently harbor resistance to cysteine protease inhibitors due to selection by other antimalarial agents. We cannot yet comment on the likelihood that the use of cysteine protease inhibitors as antimalarials might select for parasites resistant to these agents. Attempts to select for cysteine protease inhibitor-resistant parasites are under way.

In summary, we have demonstrated that falcipain-2 is highly conserved among different strains of *P. falciparum*. In addition, parasite strains that vary greatly in geographic origin and sensitivity to standard antimalarial drugs were equally sensitive to a set of potent falcipain-2 inhibitors. Thus, results from studies

TABLE 2. Sensitivities of five *P. falciparum* strains to different falcipain-2 inhibitors

Compound[a]	IC_{50} (nM) based on parasite development (metabolism) assay[b]				
	D6	Dd2	HB3	ItG	W2
Mu-Phe-Hph-CH_2F	0.90 (8.55)	0.78 (4.87)	0.66 (3.38)	0.51 (9.18)	0.81 (8.36)
Mu-Leu-HphVSPh	1.51 (26.6)	0.66 (13.0)	1.60 (15.5)	1.03 (29.2)	1.20 (28.6)
N-Me-pipu-Leu-HphVSPh	0.58 (9.93)	0.40 (2.57)	0.44 (2.87)	0.60 (10.5)	0.45 (5.21)
N-Me-pipu-Leu-HphVS-2Np	0.22 (4.72)	0.32 (4.36)	0.19 (1.55)	0.18 (2.02)	0.20 (2.74)
Chloroquine	1.74 (7.89)	26.0 (158)	1.40 (16.0)	38.1 (153)	17.7 (123)

[a] Mu-Phe-Hph-CH_2F, morpholine urea-phenylalanine-homophenylalanine-fluoromethyl ketone; Mu-Leu-HphVSPh, morpholine urea-leucine-homophenylalanine-phenyl vinyl sulfone; *N*-Me-pipu-Leu-HphVSPh, *N*-methyl piperazine urea-leucine-homophenylalanine-phenyl vinyl sulfone; *N*-Me-pipu-Leu-HphVS-2Np, *N*-methyl piperazine urea-leucine-homophenylalanine-naphthalene vinyl sulfone.

[b] IC_{50}s, determined from dose response curves, are shown for assays of parasite development (based on parasite counts) and metabolism (based on [^{3}H]hypoxanthine uptake) performed as described in the text.

of cysteine protease inhibitors with any *P. falciparum* strain are probably representative of any natural infection. These data support continued efforts toward the development of inhibitors of falcipain-2 as new antimalarial agents.

Nucleotide sequence accession numbers. Nucleotide sequence data have been deposited in the GenBank database with accession numbers AF282975 to AF282979.

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