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Antimalarial Activities of Novel Synthetic Cysteine Protease Inhibitors

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Among promising new targets for antimalarial chemotherapy are the cysteine protease hemoglobinases falcipain-2 and falcipain-3. We evaluated the activities of synthetic peptidyl aldehyde and α-ketoamide cysteine protease inhibitors against these proteases, against cultured *Plasmodium falciparum* parasites, and in a murine malaria model. Optimized compounds inhibited falcipain-2 and falcipain-3, blocked hemoglobin hydrolysis, and prevented the development of *P. falciparum* at nanomolar concentrations. The compounds were equally active against multiple strains of *P. falciparum* with varied sensitivities to standard antimalarial agents. The peptidyl inhibitors were consistently less active against vinckepain-2, the putative falcipain-2 and falcipain-3 ortholog of the rodent malaria parasite *Plasmodium vinckei*. The lead compound morpholinocarbonyl-leucine-homophenylalanine aldehyde, which blocked *P. falciparum* development at low nanomolar concentrations, was tested in a murine *P. vinckei* model. When infused continuously at a rate of 30 mg/kg of body weight/day, the compound delayed the progression of malaria but did not eradicate infections. Our data demonstrate the potent antimalarial activities of novel cysteine protease inhibitors. Additionally, they highlight the importance of consideration of the specific enzyme targets of animal model parasites. In the case of falcipains, differences between *P. falciparum* and rodent parasites complicate the use of the rodent malaria model in the drug discovery process.

Malaria remains one of the most important infectious disease problems in the world (2). The treatment and control of malaria are greatly limited by the increasing resistance of malaria parasites, particularly *Plasmodium falciparum*, to available drugs (7). New antimalarial agents, ideally directed against new targets, are therefore an urgent priority (5).

Among the potential targets for drugs directed against *P. falciparum* are proteases that hydrolyze hemoglobin to provide amino acids for parasite protein synthesis. Multiple proteases appear to participate in this process (3, 8), including the cysteine proteases falcipain-2 (15) and falcipain-3 (17). Inhibitors of these cysteine proteases block the hydrolysis of hemoglobin and thereby halt the development of cultured *P. falciparum* parasites (10, 13). Efforts are therefore under way to discover inhibitors of falcipain-2 and falcipain-3 with acceptable properties for new antimalarial drugs.

Antimalarial drug discovery routinely includes in vivo efficacy studies of mice infected with rodent malaria parasites, as *P. falciparum* can be maintained only in a few species of primates which are in very limited supply. Mouse models have facilitated the development of a number of antimalarial drugs, but they may have limitations when drug targets in *P. falciparum* and rodent parasites differ. In the case of cysteine proteases, a single homolog of falcipain-2 and falcipain-3 has been identified in four species of rodent malaria parasites (12) and biochemically characterized for *Plasmodium vinckei* (19). The homolog vinckepain-2 is quite similar to falcipain-2 and falcipain-3 (about 50% sequence identity), but it differs in some important respects, including the kinetics of the hydrolysis of peptide substrates (19).

Peptidyl cysteine protease inhibitors have previously demonstrated antimalarial activities in vitro (11, 13) and in vivo (6, 9), although in vivo activities have not been as robust as might have been anticipated based on the in vitro findings. One explanation for this limitation in activity might be the differences in actions against *P. falciparum* and rodent parasite targets. To evaluate the antimalarial properties of a new class of cysteine protease inhibitors and to consider the impact of the different parasite targets in drug efficacy studies, we have evaluated the protease inhibitory activities and in vitro and in vivo antimalarial activities of peptidyl aldehyde and α-ketoamide inhibitors.

MATERIALS AND METHODS

Synthesis of cysteine protease inhibitors. The synthesis of peptidyl aldehydes (20) and α-ketoamides (14) was accomplished essentially as previously described (20). Synthetic details of individual compound synthesis were as previously described (M. Lim-Wilby, J. E. Semple, G. L. Araldi, E. A. Goldman, and M. I. Weinhouse, 20 June 2000, Patent Cooperation Treaty application WO02/48097A1).

Parasites. *P. falciparum* parasites of the strains indicated in Results were cultured with human erythrocytes (2% hematocrit) in RPMI medium and 10% human serum (11). Parasites were synchronized by serial treatments with 5% D-sorbitol (4). For in vivo experiments, Swiss Webster mice were infected by intraperitoneal injection with frozen stocks of *P. vinckei*-infected murine erythrocytes. Parasites were then passaged in mice by the intraperitoneal injection of *P. vinckei*-infected erythrocytes. Parasitemias of cultures and in infected mice were determined from Giemsa-stained blood smears.

Protease inhibition assays. Activity was measured as hydrolysis of the fluorogenic substrate benzyloxycarbonyl-Leu-Arg-7-amino-4-methyl-coumarin (Z-Leu-Arg-AMC) by using microplate reactions and a Fluoroskan II spectrofluorom-

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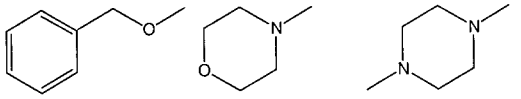
TABLE 1. Activities against native falcipain-2 and cultured *P. falciparum* parasites[a]



Compound	Structure	IC_{50} for falcipain-2 (nM)[b]	Inhibition of parasite development (nM)		
			IC_{50}	IC_{90}	FV abnl
9035	Z-Leu-Tyr-al	4	40	600	100
9036	Z-Phe-Tyr-al	16	70	>1,000	1,000
9037	Z-Leu-hPhe-al	2	30	500	100
9038	Z-Phe-hPhe-al	20	300	>1,000	1,000
9039	$PhSO_2$-Leu-hPhe-al	6	100	700	100
9040	$PhSO_2$-Phe-hPhe-al	31	400	>1,000	1,000
9042	$PhSO_2$-Leu-Tyr-al	9	30	400	100
9043	$PhSO_2$-Phe-hPhe-al	91	700	>1,000	>1,000
9044	Phe-hPhe-(CO)-Phe-NH_2	1,500	ND	ND	ND
9045	Morpholino-CO-Leu-hPhe-(CO)-Phe-NH_2	1	10	70	100
9046	Morpholino-CO-Leu-Tyr-al	3	4	400	100
9047	Morpholino-CO-Phe-Tyr-al	33	100	1,000	1,000
9048	4-Methylpiperazinyl-CO-Phe-Tyr-al	33	ND	ND	ND
9050	Morpholino-CO-Leu-hPhe-al	1	10	70	10
9051	Morpholino-CO-Phe-hPhe-al	9	10	500	100
9052	4-Methylpiperazinyl-CO-Phe-hPhe-al	60	ND	ND	ND

[a] For IC_{50} calculations, goodness of fit was documented by R^2 values that were generally >0.95. Abbreviations: Ph, phenyl; hPhe, homophenylalanine; al, aldehyde; FV abnl, lowest concentration (among serial 10-fold dilutions) at which a food vacuole abnormality, indicative of a block in hemoglobin degradation, was seen in the majority of parasites; ND, not done.

[b] Based on means from three assays comparing activities of compounds and solvent controls against Z-Leu-Arg-AMC.

eter, as described previously (11). For inhibitor assays, trophozoite extracts (in 0.1 M sodium acetate) or recombinant proteases (prepared as described previously [15, 16, 19]) were incubated with inhibitors (added from stocks concentrated 100- to 1,000-fold in dimethyl sulfoxide [DMSO]) with 10 mM dithiothreitol, pH 5.5, for 30 min at room temperature before the substrate (50 μM) was added. Equal concentrations of enzymes were used for each experiment. Multiple inhibitor concentrations were studied in duplicate or triplicate in multiple experiments, and the rates of hydrolysis were determined and compared to those of controls containing equal concentrations of DMSO. All values were normalized to the control activity, and 50% inhibitory concentrations (IC_{50}s) were calculated with the Prism 3.0 program (GraphPad Software), with data being fitted by nonlinear regression, as previously described (18).

Inhibition of cultured malaria parasites. Microwell cultures of synchronized parasites were incubated with inhibitors (from stocks concentrated 100- to 1,000-fold in DMSO) or chloroquine (Sigma) for 48 h beginning at the ring stage. Culture medium was changed after 24 h, with maintenance of the appropriate inhibitor concentration. After 48 h, when control cultures contained nearly all new ring-stage parasites, Giemsa-stained smears were made, the numbers of new rings per 1,000 erythrocytes were counted, and counts of treated cultures were compared with those from controls containing an equal concentration of DMSO. All values were normalized to the control activity, and IC_{50}s and IC_{90}s were calculated with the Prism 3.0 program as described above.

Evaluation of in vivo antimalarial activity. In the initial experiments, mice were infected with 10^5 *P. vinckei*-infected erythrocytes passaged from another infected mouse, and the courses of infection in mice treated with intraperitoneal injections of compound 9050 (Table 1) and in controls receiving sham injections of solvent were compared. In subsequent studies, mice underwent implantation of Alzet subcutaneous infusion pumps (Alza) containing compound 9050 in 12.5% Cremophor RH40 (BASF) set to be administered at a rate of 30 mg/kg of body weight/day by continuous infusion. Controls underwent implantation of pumps containing only solvent. One day after infusion, 10^5 *P. vinckei*-infected erythrocytes were injected intraperitoneally. The courses of infection in treated and control mice were then monitored by daily clinical evaluation and the counting of parasites in Giemsa-stained smears that were obtained from tail snips. Euthanasia was performed when parasitemias exceeded 50%.

RESULTS

Inhibition of native plasmodial cysteine proteases. In the initial studies, we evaluated the inhibition of the cysteine protease activities of soluble extracts of *P. falciparum*. It is now appreciated that multiple cysteine proteases are expressed by *P. falciparum* but that over 90% of the activity of the extracts measured with the substrate Z-Leu-Arg-AMC is that of falcipain-2 (15). Many low- to mid-nanomolar-range inhibitors of the cysteine protease activity were identified (Table 1). As seen previously with other classes of inhibitors, compounds with Leu at the P2 position were consistently more effective than those with Phe at P2 (9, 11). In this regard, falcipain-2 differs from the host cysteine proteases cathepsin L and B and many other papain family cysteine proteases (1).

Inhibition of recombinant plasmodial cysteine proteases. Recombinant forms of the *P. falciparum* cysteine proteases falcipain-2 and falcipain-3 and of the *P. vinckei* homolog vinckepain-2 are now available. All of these enzymes were expressed in *Escherichia coli* and refolded to active forms, as previously described (15, 16, 19). Activities of four potent inhibitors from our initial screen were tested against the recombinant plasmodial proteases (Table 2). Nanomolar-level inhibition of the *P. falciparum* proteases was seen with each inhibitor. As noted against native protease, inhibitors with P2 Leu were most active. Although similarly active against falcipain-2 and falcipain-3, the compounds were much less effective against vinck-

TABLE 2. Inhibition of recombinant plasmodial cysteine proteases

Compound	IC_{50} (nM)		
	Falcipain-2	Falcipain-3	Vinckepain-2
9035	9.3	15.4	91.9
9045	1.3	2.4	12.5
9050	3.0	3.6	42.1
9051	50.9	67.8	>1,000

TABLE 3. Activity against different strains of cultured *P. falciparum* parasites

Compound	IC_{50} (nM)				
	W2	ItG	D6	Dd2	HB3
9037	8.2	19	12	19	16
9045	2.9	5.3	2.5	2.4	3.1
9050	1.1	0.96	1.4	1.9	2.6

epain-2, particularly when the compound contained a Phe at P2 (Table 2).

Inhibition of cultured malaria parasites. Compounds with submicromolar levels of activity against native falcipain-2 were incubated with synchronous populations of cultured *P. falciparum* parasites (W2 strain) for 48 h, and parasitemia was then assessed microscopically to compare the parasite development in treated cultures with that in control cultures. Multiple inhibitors exhibited potent antimalarial effects (Table 1). Inhibition of parasite development by the falcipain inhibitors correlated with activity against falcipain-2. In addition, inhibition of development was consistently accompanied by the presence of swollen, darkly staining parasite food vacuoles, consistent with a block in the hydrolysis of hemoglobin (10).

Antimalarial chemotherapy is currently challenged by the emergence of parasites that are resistant to multiple classes of antimalarials. New antimalarials should ideally be active against all strains of *P. falciparum*, including multidrug-resistant strains. We tested the activities of three compounds against five strains of *P. falciparum* that differ widely in their levels of resistance to other agents (Table 3). Three of the strains, W2 (which was used for all other described experiments), ItG, and Dd2, are resistant to multiple antimalarials (18). The three tested compounds had very similar activities against the five strains of *P. falciparum*, with no evidence that the activities against multidrug-resistant strains differed from those against broadly sensitive strains.

In vivo antimalarial activity of an aldehyde cysteine protease inhibitor. Compound 9050 exhibited excellent activity against falcipains and against cultured parasites. We assessed the in vivo activity of this compound in *P. vinckei*-infected mice. In the initial trials, compound 9050 showed antimalarial activity when it was administered intraperitoneally to *P. vinckei*-infected mice, but the activity was fairly low, with only minor differences between the progression of malaria in treated animals and that in control animals (data not shown). Limitations in activity were likely due, in part, to a short half-life of the compound. An intraperitoneal dose of 50 mg/kg in 12.5% Cremophor RH40 provided a maximum concentration of the drug in serum of 9.7 μg/ml and a half-life at β phase of 87.1 min, and a subcutaneous dose of 100 mg/kg in the same diluent provided a maximum concentration of the drug in serum of 13.0 μg/ml and a half-life at β phase of 56.2 min; in each case, values are based on means from evaluations of three animals each at 30, 60, 120, 240, and 480 min after dosage (unpublished data). In addition, the formulation of the compound was difficult due to limited solubility. To provide consistent levels in blood and improve drug delivery, mice were dosed by a subcutaneous infusion pump to provide 30 mg/kg/day by continuous infusion. Mice were infected by the intraperitoneal administration of 10^5 *P. vinckei*-infected erythroctes 1 day after the implantation of the infusion pump, and the progression of malaria infections was monitored by daily blood smears from treated mice and control mice (with sham infusion pumps). Treated mice had a consistent delay in the progression of *P. vinckei* parasitemia (Fig. 1). However, the antimalarial

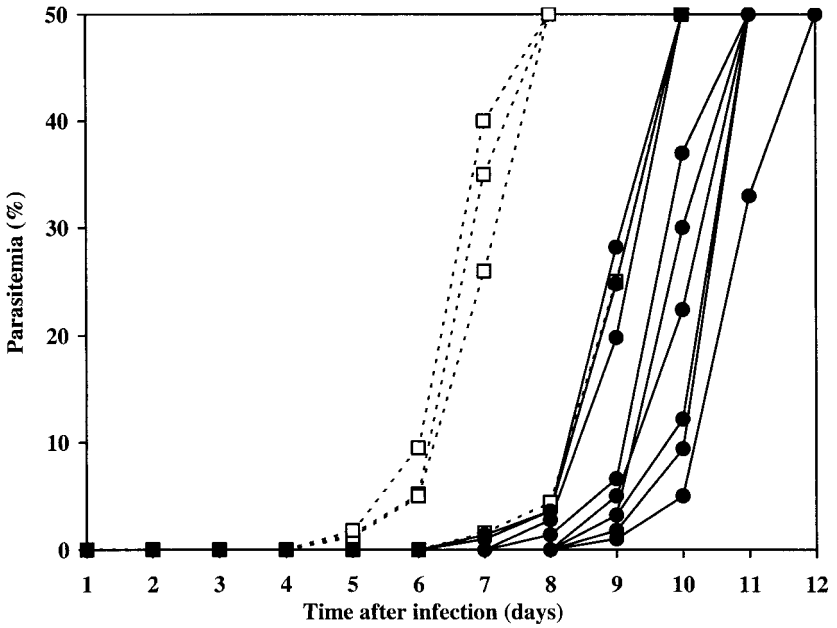


FIG. 1. Treatment of *P. vinckei*-infected mice with compound 9050. Mice received subcutaneous pumps set to administer 30 mg of compound 9050/kg/day for 14 days. After 1 day, 10^5 *P. vinckei*-infected erythrocytes were injected intraperitoneally, and the parasitemias of four control (open squares) and nine treated (filled circles) mice were monitored daily. Euthanasia was performed when parasitemias exceeded 50%.

effect was modest, and all treated and control mice eventually succumbed to lethal infections.

DISCUSSION

We have identified small-molecule aldehydes and amides as potent inhibitors of plasmodial cysteine proteases. As with older studies of fluoromethyl ketone (13) and vinyl sulfone (11) inhibitors, a consistent structure-activity relationship was seen: compounds containing Leu at the P2 position were about an order of magnitude more potent than those with Phe at this position. These results are consistent with a strong preference in falcipain-2, falcipain-3, and vinckepain-2 for peptide substrates with P2 Leu. They also identify a key difference between the plasmodial cysteine proteases and many other papain family cysteine proteases, including the abundant host proteases cathepsin B and cathepsin L (1). This result suggests the potential for a specificity for inhibitors of plasmodial enzymes, but other, more specialized host cysteine proteases (cathepsin K and cathepsin S) share the P2 Leu preference.

The inhibition of the proteolytic activities of trophozoite extracts correlated with the inhibition of parasite development. A number of low-nanomolar-concentration inhibitors of proteolytic activity blocked parasite development at low- to mid-nanomolar concentrations. Antimalarial activity was accompanied by a marked morphological abnormality, whereby the parasite food vacuole filled with undegraded hemoglobin, consistent with action against cysteine protease hemoglobinases.

Until recently, only one *P. falciparum* cysteine protease activity (termed trophozoite cysteine protease or falcipain) was identified, and older studies considered only the inhibition of this activity by potential antimalarials. However, recent advances in biochemistry and genomics have led to the identification of three fairly typical *P. falciparum* papain family cysteine proteases (12). Two of these proteases, falcipain-2 and falcipain-3, appear to be the key hemoglobinase targets. It is also now appreciated that older studies that evaluated action against the proteolytic activity of trophozoite extracts primarily evaluated falcipain-2. Considering these factors, it was of interest to evaluate the activities of lead inhibitors of both falcipain-2 and falcipain-3. These enzymes are similar in sequence (68% identity) and in substrate specificity, but they differ markedly in activity, such that falcipain-2 has much greater activity against a range of peptide substrates (17). Regarding inhibition by aldehyde and amide inhibitors, however, activities against the two enzymes were very similar. These data suggest that a single specific protease inhibitor may provide effective inhibition of both falcipain-2 and falcipain-3 and thus provide potent inhibition of hemoglobin hydrolysis and parasite development.

We have also recently characterized cysteine proteases of rodent malaria parasites that appear to be orthologs of falcipain-2 and falcipain-3. A single ortholog of the two *P. falciparum* proteases appears to be present in *P. vinckei* and three other rodent malaria parasites (12). Interestingly, the rodent parasite cysteine proteases all contain an unusual active-site substitution that, in the case of the *P. vinckei* protease vinckepain-2, mediates unusual kinetics (19). As mouse models are generally a key component of malaria drug discovery efforts, it was important to compare the inhibitor sensitivities of *P. falciparum* and *P. vinckei* cysteine proteases. In fact, vinckepain-2 was consistently less well inhibited, by about an order of magnitude, than were falcipain-2 and falcipain-3 by optimal inhibitors. Inhibition was particularly poor with a compound containing a P2 Phe, consistent with the unusually poor activity of vinckepain-2 against substrates with P2 Phe (19).

Although activity against vinckepain-2 was less marked than that against the *P. falciparum* proteases, it was of interest to test the in vivo antimalarial activity of compound 9050. Unfortunately, this compound is limited by relatively poor pharmacokinetics in mice and problems with formulation, necessitating the use of subcutaneous infusion pumps for administration. Our in vivo studies demonstrated only modest antimalarial activity for compound 9050 against *P. vinckei* malaria. However, the studies offer additional justification for the use of cysteine protease inhibitors as antimalarials and suggest that differences between *P. falciparum* and *P. vinckei* targets may contribute to the limited in vivo efficacies of some cysteine protease inhibitors. The results further suggest a need for rethinking traditional approaches. Antimalarial drug discovery has typically relied on validation with rodent models before advancement to full development. However, in cases where human and rodent parasite targets differ, it may be appropriate to bring promising compounds forward without validation in rodents, perhaps after extensive pharmacokinetic studies, and then to evaluate their efficacies in *P. falciparum* models.

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