Inhibition of a *Plasmodium vinckei* Cysteine Proteinase Cures Murine Malaria

Philip J. Rosenthal,* Garson K. Lee,* and Robert E. Smith‡
*Department of Medicine, San Francisco General Hospital and University of California, San Francisco, California 94143; and ‡Prototek, Dublin, California 94568

Abstract

Intraerythrocytic malaria parasites degrade hemoglobin as a principal source of amino acids for parasite protein synthesis. We have previously identified a *Plasmodium falciparum* trophozoite cysteine proteinase as a putative hemoglobinase and shown that specific inhibitors of this proteinase block the hydrolysis of globin and the development of cultured parasites. We now show that the murine malaria parasite *Plasmodium vinckei* has an analogous cysteine proteinase with similar biochemical properties to the *P. falciparum* proteinase, including an acid pH optimum, a preference for the peptide proteolytic substrate benzyloxycarbonyl (Z)-Phe-Arg-7-amino-4-methylcoumarin, and nanomolar inhibition by seven peptide fluoromethyl ketone proteinase inhibitors. Thus, *P. vinckei* offers a model system for the in vivo testing of the antimalarial properties of cysteine proteinase inhibitors. One of the proteinase inhibitors studied, morpholine urea (Mu)-Phe-Homophenylalanine (HPhe)-CH_2F strongly inhibited the *P. vinckei* cysteine proteinase in vitro and rapidly blocked parasite cysteine proteinase activity in vivo. When administered four times a day for 4 d to *P. vinckei*–infected mice, Mu-Phe-HPhe-CH_2F elicited long-term cures in 80% of the treated animals. These results show that peptide proteinase inhibitors can be effective antimalarial compounds in vivo and suggest that the *P. falciparum* cysteine proteinase is a promising target for chemotherapy. (*J. Clin. Invest.* 1993. 91:1052–1056.) Key words: chemotherapy • peptide • proteinase inhibitor • hemoglobin

Introduction

Malaria remains one of the most important infectious diseases in the world, in part due to the increasing resistance of malaria parasites to available drugs (1). There is thus an urgent need to identify new targets for antimalarial chemotherapy and to develop model systems for the testing of potential antimalarial compounds directed against these targets. Among potential new chemotherapeutic targets are enzymes responsible for the degradation of hemoglobin by intraerythrocytic malaria parasites. These parasites degrade host erythrocyte hemoglobin as a principal source of amino acids for parasite protein synthesis (2). This process involves the transport of erythrocyte cytoplasm to acidic food vacuoles, where the heme component of hemoglobin is processed into hemozoin, and globin is hydrolized to free amino acids (2). As is the case with mammalian lysosomes, malarial food vacuoles appear to contain both cysteine (3) and aspartic (4–6) proteinases, both of which may participate in the hydrolysis of globin (7).

We previously identified a cathepsin L–like *Plasmodium falciparum* trophozoite cysteine proteinase as a putative hemoglobinase (3). On evaluation of a panel of fluoromethyl ketone proteinase inhibitors, inhibition of the trophozoite cysteine proteinase correlated with the inhibition of both hemoglobin degradation and parasite development (8). Thus, it appeared that the antimalarial effects of the inhibitors were due specifically to the inhibition of the trophozoite cysteine proteinase, and therefore that this proteinase is an essential hemoglobinase. Furthermore, benzyloxycarbonyl (Z)-Phe-Arg-CH_2F, the most effective inhibitor studied, had very potent antimalarial effects, with an IC_{50} for parasite killing of 64 nM (8).

To assess the feasibility of using peptide proteinase inhibitors as antimalarials in vivo, and, more generally, to determine whether the malarial cysteine proteinase might be an appropriate target for chemotherapy, we have studied the proteolytic activity of the murine malaria parasite *Plasmodium vinckei.* We now show that *P. vinckei* has cysteine proteinase activity similar to that of *P. falciparum,* and that the fluoromethyl ketone inhibitor morpholine urea (Mu)-Phe-Homophenylalanine(Hphe)-CH_2F strongly inhibited proteolytic activity in vitro and in vivo. When administered four times a day for 4 d to *P. vinckei*–infected mice, Mu-Phe-HPhe-CH_2F elicited long-term cures in 80% of the treated animals. Our results add in vivo support to our prior in vitro observations suggesting that the cysteine proteinases of malaria parasites may be useful targets for chemotherapy.

Methods

Parasite culture. Itg2 strain *P. falciparum* parasites were cultured in human erythrocytes (from which leukocytes and platelets were removed) as previously described (9, 10). Synchrony was maintained with sorbitol (11). Frozen stocks of *P. vinckei* (kindly provided by Dr. William Weidanz, University of Wisconsin) were used to infect Balb c mice by intraperitoneal injection and parasites were subsequently passaged in mice. Parasites were evaluated with Giemsa-stained smears of blood obtained from tail vein incisions.

Preparation of parasite extracts. Parasite-infected erythrocytes obtained from *P. falciparum* cultures (at ~ 10% parasitemia) or *P. vinckei*–infected mouse blood (usually at 30–50% parasitemia) were washed in PBS, incubated with 0.1% saponin in PBS to lyse erythrocyte membranes, and washed three times with ice-cold PBS. Parasite proteins were then solubilized with two freeze-thaw cycles in water and the supernatant after centrifugation (13,000 *g*, 10 min, 4°C) was collected. Extracts of uninfected erythrocytes were prepared in an identical manner.

Proteinase inhibitors. Leupeptin and L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) were purchased from Sigma Immunochemicals, St. Louis, MO. All peptide fluoromethyl ketones were generously provided by Prototek, Inc., Dublin, CA. The fluoromethyl ketones were solubilized in DMSO before dilution in aqueous buffers for proteolytic activity assays.

Address correspondence to Philip Rosenthal, Box 0811, University of California, San Francisco, CA 94143.
Received for publication 1 June 1992.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/93/03/1052/05 $2.00
Volume 91, March 1993, 1052–1056

Proteolytic activity assays. Gelatin-substrate PAGE was performed as previously described (12). In brief, this technique involves electrophoresis of nonreduced proteins on a gelatin-containing gel, removal of SDS from the gel by washing with 2.5% Triton X-100, overnight incubation (0.1 M sodium acetate, 10 mM dithiothreitol, pH 6.0, 37°C) of the gel to allow hydrolysis of the gelatin by renatured proteinases, and staining with Coomassie blue. Proteinases are identified as clear bands in the blue-staining gel. To evaluate the effects of proteinase inhibitors, the inhibitors were incubated with parasite extracts (1 h, room temperature) before samples were mixed with electrophoresis sample buffer, and they were included in the overnight gel incubation buffer. To evaluate the pH optimum of proteolytic activity the buffer used for overnight incubation of the gel was varied (pH 3.0–6.0, sodium acetate; pH 6.0–8.0, sodium phosphate; pH 8.0–10.0, Tris HCl). Proteolytic activity was also measured with two other substrates: (*a*) fluorogenic peptide substrates containing the 7-amino-4-methyl-coumarin (AMC)[1] detecting group (Enzyme Systems Products, Dublin, CA) and (*b*) [^{14}C]-methemoglobin (Dupont New England Nuclear, Wilmington, DE), both as previously described (3). The inhibition of human cathepsin B and cathepsin L (Enzyme Systems Products) was determined with the substrate Z-Phe-Arg-AMC in an identical fashion to that used for parasite extracts.

Evaluation of in vivo effects of Mu-Phe-Hphe-CH_2F. Mice infected with high parasitemias of *P. vinckei* (generally 10–40%) were treated subcutaneously with two 100 mg/kg doses of Mu-Phe-Hphe-CH_2F in DMSO at 6-h intervals. At zero h, and at 2, 4, 6, and 8 h after the initial injection mice were killed, parasite proteins were extracted as discussed above, and the proteolytic activity of the extracts was determined using the fluorogenic substrate Z-Phe-Arg-AMC. To confirm that the zero time point was a representative positive control, DMSO-treated control mice were also sacrificed at 2, 4, 6, and 8 h, and the proteolytic activity of their parasites was measured. Activities of the control mice were equivalent throughout the 8-h period of the experiment.

Evaluation of in vivo antimalarial efficacy of fluoromethyl ketones. In initial experiments mice were treated with intraperitoneal or subcutaneous doses of fluoromethyl ketones in DMSO 4–6 h after intraperitoneal infection with *P. vinckei.* These experiments identified Mu-Phe-Hphe-CH_2F as the most effective antimalarial compound in vivo. In subsequent experiments mice were infected intraperitoneally with 10^6 *P. vinckei* parasites and followed for 3–4 d until parasitemias of ~ 1.0% had developed. Therapy was then instituted with subcutaneous 100 mg/kg doses of Mu-Phe-Hphe-CH_2F 2–4 times per day for 4 d. Controls were treated in an identical manner with the volume of DMSO (50 μl) in which Mu-Phe-Hphe-CH_2F was solubilized. Parasitemias were assessed daily. In most experiments mice were sacrificed when parasitemias were above ~ 30% and rapidly increasing, but in one experiment 10 mice treated four times per day were followed through the development of significant parasitemias as described in Results. Treated mice, untreated infected controls, and treated uninfected controls were observed daily for evidence of toxicity.

Results

Identification and characterization of a P. vinckei cysteine proteinase. Extracts of *P. vinckei* were evaluated with gelatin substrate PAGE. The extracts had a predominant proteolytic activity at M_r 27,000, with a minor activity (possibly an active proform of the same proteinase) at M_r 36,000 (Fig. 1). The activity was completely inhibited by the cysteine proteinase inhibitor E-64 (Fig. 1) and had an acidic pH optimum of 4.0–6.0 (Fig. 2). Acid cysteine proteinase activity was also identified with two other proteolytic assays. With both the fluorogenic peptide substrate Z-Phe-Arg-AMC and the substrate [^{14}C]methemoglobin, proteolytic activity was strongly inhibited by the cysteine proteinase inhibitors leupeptin and E-64 (Fig. 3). With fluorogenic peptide substrates, the cathepsin L substrate Z-Phe-Arg-AMC was preferred to typical substrates for the related lysosomal proteinases cathepsin B (Z-Arg-Arg-AMC) and cathepsin H (Z-Arg-AMC) (Table I). With the substrate Z-Phe-Arg-AMC extracts of uninfected murine erythrocytes had < 1% of the proteolytic activity (compared as activity per cell) of parasite extracts, and no activity was seen on gelatin substrate polyacrylamide gels. The above data show that the *P. vinckei* cysteine proteinase is biochemically very similar to the *P. falciparum* trophozoite cysteine proteinase (3, 12), suggesting that it is a functional analogue of the *P. falciparum* proteinase.

Inhibition of the P. vinckei cysteine proteinase in vitro by fluoromethyl ketone proteinase inhibitors. The inhibition of *P. vinckei* cysteine proteinase activity by a number of fluoromethyl ketone peptide proteinase inhibitors was evaluated using the fluorogenic substrate Z-Phe-Arg-AMC (Table II). As for the *P. falciparum* trophozoite cysteine proteinase (8), a number of compounds inhibited the *P. vinckei* proteinase at nanomolar concentrations. However, the profile of inhibition was not identical to that of the *P. falciparum* cysteine proteinase. The most effective inhibitor of the *P. vinckei* proteinase was Mu-Phe-Hphe-CH_2F, with an IC_{50} of 5.1 nM. Using the gelatin gel assay, Z-Phe-Arg-CH_2F and Mu-Phe-Hphe-CH_2F

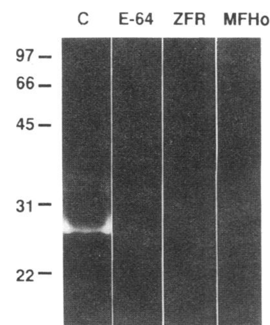

Figure 1. Inhibition of the *P. vinckei* cysteine proteinase by proteinase inhibitors. Gelatin-substrate PAGE (12.5% polyacrylamide gel) of *P. vinckei* proteins was performed. For the control parasite extract (*C*), the clear band at M_r 27,000 and the weaker band at M_r 36,000 represent *P. vinckei* proteinase activities. For the other lanes, equal quantities of parasite extracts were incubated with E-64 (100 μM), Z-Phe-Arg-CH_2F (*ZFR*, 1 mM), or Mu-Phe-Hphe-CH_2F (*MFHo*, 1 mM) before electrophoresis (1 h, room temperature) and gel slices were incubated with the inhibitors (at pH 6.0) during the overnight incubation that preceded gel staining with Coomassie blue. Molecular weight standards are indicated in kilodaltons. Proteinase activity was strongly inhibited by all three proteinase inhibitors.

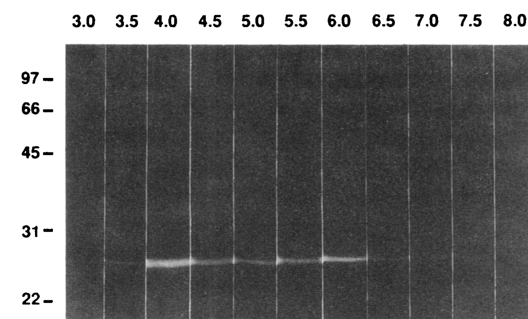

Figure 2. pH profile for proteinase activity. Equal quantities of *P. vinckei* extracts were electrophoresed on gelatin substrate PAGE gels. After electrophoresis the gel lanes were incubated overnight in buffers at the indicated pH. Peak activities at M_r 27,000 and M_r 36,000 were seen at pH 4.0–6.0.

1. *Abbreviation used in this paper:* AMC, 7-amino-4-methyl-coumarin.

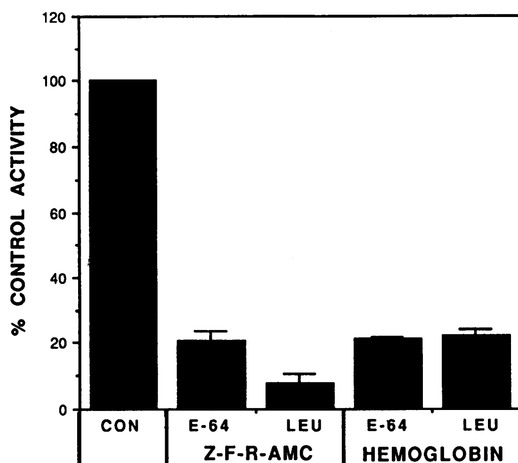

Figure 3. Inhibition of *P. vinckei* proteolytic activity by cysteine proteinase inhibitors. The proteolytic activities of extracts of *P. vinckei* were determined in the presence of no inhibitor (*CON*), E-64 (100 μM), or leupeptin (*LEU*, 100 μM) using either the fluorogenic peptide substrate Z-Phe-Arg-AMC (*Z-F-R-AMC*) or the radioactive substrate [^{14}C]methemoglobin (*HEMOGLOBIN*). Both assays were performed in 0.1 M sodium acetate, 10 mM dithiothreitol, pH 6.0. Results are presented as the mean percentage of control activity. Error bars represent the standard deviations of results performed in triplicate.

Table II. Inhibitor Sensitivity of P. falciparum and P. vinckei Cysteine Proteinases

Compound	*P. falciparum* IC_{50}	*P. vinckei* IC_{50}
	nM	*nM*
Z-Phe-Arg-CH_2F	0.36	33
Z-DPhe-Arg-CH_2F	14	110
Z-Phe-Ala-CH_2F	28	90
Z-Phe-Phe-CH_2F	50	ND*
Z-DPhe-Ala-CH_2F	1300	2200
Mu-Phe-HPhe-CH_2F	3.0	5.1
Mu-Leu-HPhe-CH_2F	0.42	15
Mu-Phe-Arg$(NO)_2$-CH_2F	2.4	110
Mu-$(TyrOMe)_2$-HPhe-CH_2F	8.5	18

Soluble extracts of *P. falciparum* and *P. vinckei* were prepared, and proteolytic activity was assayed with the fluorogenic peptide substrate Z-Phe-Arg-AMC. The activity of control samples was compared to activities in the presence of multiple concentrations of the fluoromethyl ketones listed. For each inhibitor, the concentration at which activity was inhibited 50% (IC_{50}) was calculated. The results for the first five inhibitors with *P. falciparum* extracts were reported previously (8). D represents the D-stereoisomer of the amino acid following this symbol. * ND, not done.

specifically inhibited the *P. vinckei* M_r 27,000 cysteine proteinase (Fig. 1). These inhibitors were not specific for only parasite proteinases, however, as Z-Phe-Arg-CH_2F (8) and Mu-Phe-Hphe-CH_2F also inhibited host proteinases at nanomolar concentrations. For Mu-Phe-Hphe-CH_2F the IC_{50} for cathepsin L was 3.1 nM and for cathepsin B was 3.0 nM.

Inhibition of the P. vinckei cysteine proteinase in vivo by fluoromethyl ketone proteinase inhibitors. To study the in vivo effectiveness of fluoromethyl ketones against murine malaria, mice were infected intraperitoneally with *P. vinckei,* fluoromethyl ketones were administered intraperitoneally or subcutaneously, and daily parasitemias were compared with those of untreated controls. The most effective compound in preliminary studies was Mu-Phe-Hphe-CH_2F, so further therapeutic trials were instituted with this compound. To test the effectiveness of Mu-Phe-Hphe-CH_2F at inhibiting *P. vinckei* cysteine proteinase activity in vivo, mice infected with high (generally 10–40%) parasitemias of *P. vinckei* were treated with Mu-Phe-Hphe-CH_2F, and the proteolytic activity of parasite extracts from treated and control mice was compared (Fig. 4). Mu-Phe-Hphe-CH_2F blocked the proteolytic activity of *P. vinckei* by > 90% at 2, 4, and 6 h after treatment.

To test the antimalarial efficacy of Mu-Phe-Hphe-CH_2F,

Table I. Substrate Preferences of P. falciparum and P. vinckei Proteinases

Substrate	Relative activity	
	P. falciparum	*P. vinckei*
Z-PHE-ARG-AMC	100	100
Z-ARG-ARG-AMC	8.5	20.4
Z-ARG-AMC	1.6	5.0

The rate of hydrolysis of equal concentrations (50 μM) of three different peptide substrates by *P. falciparum* and *P. vinckei* extracts was measured spectrofluorometrically. The rate of hydrolysis is reported as the percentage of maximum activity. Results represent the means of two experiments for *P. falciparum* and three experiments for *P. vinckei.*

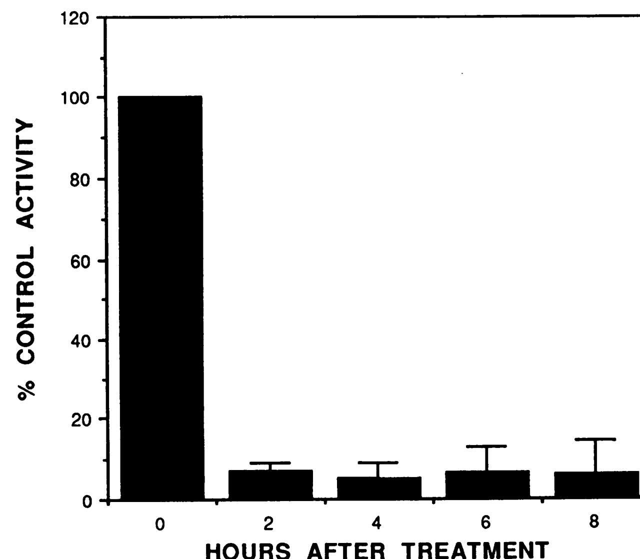

Figure 4. Inhibition of proteinase activity in vivo by Mu-Phe-Hphe-CH_2F. Mice infected with high (generally 10–40%) parasitemias of *P. vinckei* were treated with two doses of Mu-Phe-Hphe-CH_2F (100 mg/kg subcutaneously) 6 h apart. At 0 h, and at 2, 4, 6, and 8 h after the initial dose, parasites were harvested, extracts were prepared, and the proteolytic activity of the extracts was determined with the fluorogenic substrate Z-Phe-Arg-AMC. Results represent the means of duplicate experiments; error bars represent the standard deviations. The inhibitor blocked the proteolytic activity of *P. vinckei* by > 90% at 2, 4, 6, and 8 h after the initial treatment.

mice with *P. vinckei* parasitemias of ~ 1.0% were treated subcutaneously with Mu-Phe-Hphe-CH_2F for 4 d. At all dosing intervals studied, 100 mg/kg doses of the fluoromethyl ketone markedly inhibited the development of *P. vinckei* infection as compared to controls (Fig. 5). After therapy was completed, however, mice treated with two doses per day rapidly developed significant parasitemias. Mice treated with three or four doses per day remained nearly parasite-free for ~ 1 wk (5–11 d), but increasing parasitemias were eventually seen in nearly all animals. 10 mice treated four times per day were followed through this second wave of parasitemia. Of these 10 mice, one remained parasite free after treatment. The other nine mice developed measurable parasitemias 6–12 d after the completion of therapy, but all of the mice began to clear parasites after reaching peak parasitemias of 18–64%. Two of these mice died at times of decreasing parasitemia (19 and 26% after peaks of 58 and 53%, respectively), and the other seven mice rapidly cleared all parasites. On long-term followup, all eight surviving mice remained well without evidence of recurrent malaria 75 d after initial infection. In summary, 8 of 10 mice treated with Mu-Phe-Hphe-CH_2F four times per day were cured of their infections.

The second wave of parasitemia seen in the therapeutic trials was apparently due to the growth of small numbers of parasites that survived initial therapy. Analysis of parasite extracts from animals experiencing a second wave of parasitemia after therapy showed that the sensitivity of the *P. vinckei* cysteine proteinase to Mu-Phe-Hphe-CH_2F was unchanged from that seen in parasites from untreated animals (mean IC_{50} for Mu-Phe-Hphe-CH_2F was 4.4 nM for five mice). Most of the mice treated with four doses per day successfully eradicated the second wave of parasites without further therapy, presumably due to immunological mechanisms. To test this hypothesis, we reinfected five cured mice (10^6 parasites injected intraperitoneally) 50 d after they had cleared their prior parasitemias. Four of the five mice rapidly cleared their parasites after reaching peak parasitemias of 0.3–9.5%, while one mouse developed lethal parasitemia. The four immune mice then remained well and parasite free over a 6-wk period of observation.

Moderate toxicity was seen with subcutaneous administration of 100 mg/kg doses of Mu-Phe-Hphe-CH_2F, perhaps due to the inhibition of host cysteine proteinases. After a few doses of Mu-Phe-Hphe-CH_2F mice became quite lethargic; this finding resolved rapidly after the completion of therapy. Some mice developed skin ulcers at the site of multiple subcutaneous injections; these healed within 1–2 wk after the completion of therapy. No deaths were attributed to the administration of Mu-Phe-Hphe-CH_2F in the experiments discussed above.

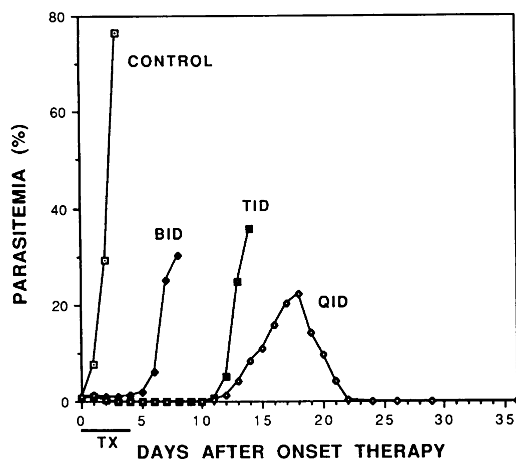

Figure 5. Treatment of murine malaria with Mu-Phe-Hphe-CH_2F. Mice were infected intraperitoneally with 10^6 *P. vinckei* parasites and then followed for 3–4 d until they had reached parasitemias of ~ 1%. The mice were then treated with Mu-Phe-Hphe-CH_2F (or, for control mice, DMSO) subcutaneously two to four times per day for 4 d (indicated by the bar labeled TX) and parasitemias were followed daily during and after treatment. Mean parasitemias for each day are shown. For mice treated with two (*BID*, $n = 3$) or three (*TID*, $n = 7$) doses per day, the experiment was terminated when mice developed rapidly increasing parasitemias after treatment. For mice treated four times per day (*QID*, $n = 10$) the experiment was continued through the rapidly increasing parasitemias. Most (8:10) of these mice were observed to clear their parasitemias and show a long-term cure.

Discussion

We have identified a cysteine proteinase of the murine malaria parasite *P. vinckei.* This proteinase is biochemically similar, in terms of its molecular mass, pH optimum, substrate specificity, and inhibitor sensitivity, to a cysteine proteinase of *P. falciparum* that we have previously identified. We hypothesize that the *P. vinckei* cysteine proteinase has the same biological role as that proposed for the *P. falciparum* proteinase, that is the hydrolysis of globin in acidic food vacuoles. *P. vinckei* does not provide an exact model for hemoglobin degradation by *P. falciparum* in two important respects. First, the *P. vinckei* proteinase differs somewhat from its *P. falciparum* analogue, as indicated by differences between the enzymes in their sensitivities to some fluoromethyl ketone inhibitors. Second, the parasites differ biologically in that *P. falciparum* degrades hemoglobin in a single large food vacuole while murine Plasmodia perform this process in multiple submicroscopic structures (13). Despite its limitations, however, the *P. vinckei* system provides a useful model for the in vivo evaluation of cysteine proteinase inhibitors as antimalarial drugs.

In an evaluation of a number of fluoromethyl ketones, Mu-Phe-Hphe-CH_2F was most effective at inhibiting the *P. vinckei* cysteine proteinase in vitro and in vivo. A single subcutaneous dose of Mu-Phe-Hphe-CH_2F strongly inhibited *P. vinckei* cysteine proteinase activity. Therapy with two to four doses per day of Mu-Phe-Hphe-CH_2F markedly inhibited parasite development during therapy of *P. vinckei*–infected mice, and a 4-d, 16 dose subcutaneous treatment course of Mu-Phe-Hphe-CH_2F cured 80% of infected mice. While Mu-Phe-Hphe-CH_2F dramatically inhibited the development of *P. vinckei* infection during therapy, however, it permanently eradicated malarial infections only after a second wave of parasitemia. Apparently the treated mice were, by virtue of the delay in infection engendered by the cysteine proteinase inhibitor, able to mount an effective immune response against infecting organisms. Such a rapid clearance of parasites by immune mice, termed "crisis," has previously been correlated with cell-mediated immunity, and requires tumor necrosis factor, interferon γ, and other yet unidentified factors (14, 15). The cured mice developed long lasting immunity; upon rechallenge of cured mice 75 d after the initial infection, four of five mice rapidly cleared their infections. Thus, the long-term protection of mice from lethal malaria in our studies probably required both the antiparasitic

effects of Mu-Phe-Hphe-CH_2F and an effective immunological response against the few parasites that avoided killing by this compound.

The use of peptides in therapy has an important theoretical limitation, as administered peptides are presumably subject to proteolysis by serum, tissue, and/or gut proteinases. Our results do suggest that the serum half-life of Mu-Phe-Hphe-CH_2F was short, as rather high doses of the compound were required for antimalarial efficacy, and as increasing the frequency of drug administration markedly improved the compound's efficacy. However, at the four dose per day regimen, Mu-Phe-Hphe-CH_2F cured 80% of mice, proving that peptides can be effective in vivo. These results suggest that further studies of the antimalarial efficacy of peptide proteinase inhibitors are warranted, but that more promising results may come from studies of nonpeptide inhibitors.

A second theoretical limitation of Mu-Phe-Hphe-CH_2F is that the compound is an effective inhibitor of host proteinases in addition to its effects on the *P. vinckei* cysteine proteinase. Indeed, some nonlethal toxicity of Mu-Phe-Hphe-CH_2F was observed, perhaps due to the inhibition of host cysteine proteinases. Treated mice did develop effective antimalarial immunity, however, suggesting that antigen processing was not adversely affected by the cysteine proteinase inhibitor. The short course of therapy required to treat malaria may allow some host proteinase inhibition without major toxicity, but, ideally, future compounds will effectively inhibit parasite, but not host proteinases.

Despite the limitations of Mu-Phe-Hphe-CH_2F in the current studies, the inhibition of the *P. vinckei* cysteine proteinase with this compound cured otherwise lethal murine malaria infections. Since the *P. vinckei* proteinase appears to be a functional analogue of the *P. falciparum* trophozoite cysteine proteinase, the results suggest that the cysteine proteinase of *P. falciparum* trophozoites remains a promising target for antimalarial chemotherapy.

Acknowledgments

We thank Dr. William Weidanz for providing stocks of *P. vinckei* and Drs. James Leech and Joel Ernst for their critical reviews of the manuscript.

This work was supported by grants from the National Institutes of Health (AI 00870), the United Nations Development Project (UNDP)/World Bank/World Health Organization (WHO) Special Programme for Research and Training in Tropical Diseases (TDR 890499), and the Defense Advanced Research Projects Agency (MDA 972-91-J-1013).

References

1. Oaks, S. C., V. S. Mitchell, G. W. Pearson, and C. C. J. Carpenter, editors. 1991. Malaria: Obstacles and Opportunities. National Academy of Sciences Press, Washington, DC.

2. Scheibel, L. W., and I. W. Sherman. 1988. Plasmodial metabolism and related organellar function during various stages of the life-cycle: proteins, lipids, nucleic acids and vitamins. *In* Malaria: Principles and Practice of Malariology. W. H. Wernsdorfer and I. McGregor, editors. Churchill Livingstone, Edinburgh. 219–252.

3. Rosenthal, P. J., J. H. McKerrow, M. Aikawa, H. Nagasawa, and J. H. Leech. 1988. A malarial cysteine proteinase is necessary for hemoglobin degradation by *Plasmodium falciparum. J. Clin. Invest.* 82:1560–1566.

4. Sherman, I. W., and L. Tanigoshi. 1983. Purification of *Plasmodium lophurae* cathepsin D and its effects on erythrocyte membrane proteins. *Mol. Biochem. Parasitol.* 8:207–226.

5. Vander Jagt, D. L., L. A. Hunsaker, and N. M. Campos. 1986. Characterization of a hemoglobin-degrading, low molecular weight protease from *Plasmodium falciparum. Mol. Biochem. Parasitol.* 18:389–400.

6. Goldberg, D. E., A. F. G. Slater, R. Beavis, B. Chait, A. Cerami, and G. B. Henderson. 1991. Hemoglobin degradation in the human malaria pathogen *Plasmodium falciparum:* a catabolic pathway initiated by a specific aspartic protease. *J. Exp. Med.* 173:961–969.

7. Rosenthal, P. J. 1991. Proteinases of malaria parasites. *In* Biochemical Protozoology. G. H. Coombs and M. J. Norths, editors. Taylor & Francis Ltd., London. 257–269.

8. Rosenthal, P. J., W. S. Wollish, J. T. Palmer, and D. Rasnick. 1991. Antimalarial effects of peptide inhibitors of a *Plasmodium falciparum* cysteine proteinase. *J. Clin. Invest.* 88:1467–1472.

9. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science (Wash. DC).* 193:673–675.

10. Rosenthal, P. J., K. Kim, J. H. McKerrow, and J. H. Leech. 1987. Identification of three stage-specific proteinases of *Plasmodium falciparum. J. Exp. Med.* 166:816–821.

11. Lambros, C., and J. P. Vanderberg. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* 65:418–420.

12. Rosenthal, P. J., J. H. McKerrow, D. Rasnick, and J. H. Leech. 1989. *Plasmodium falciparum:* inhibitors of lysosomal cysteine proteinases inhibit a trophozoite proteinase and block parasite development. *Mol. Biochem. Parasitol.* 35:177–184.

13. Rudzinska, M. A., W. Trager, and R. S. Bray. 1965. Pinocytotic uptake and the digestion of hemoglobin in malaria parasites. *J. Protozool.* 12:563–576.

14. Cohen, S., and J. A. Deans. 1988. Specific acquired immunity in experimental malaria. *In* Malaria: Principles and Practice of Malariology. W. H. Wernsdorfer and I. McGregor, editors. Churchill Livingstone, Edinburgh. 515–557.

15. Naotunne, T. d. S., N. D. Karunaweera, G. D. Giudice, M. U. Kularatne, G. E. Grau, R. Carter, and K. N. Mendis. 1991. Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. *J. Exp. Med.* 173:523–529.