Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 633–638

BIOORGANIC & MEDICINAL CHEMISTRY

Antimalarial Effects in Mice of Orally Administered Peptidyl Cysteine Protease Inhibitors

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Received 2 September 1998

Abstract—The *Plasmodium falciparum* cysteine protease falcipain is required for the degradation of hemoglobin by erythrocytic malaria parasites. In prior studies, peptidyl inhibitors of falcipain blocked hemoglobin degradation and development by cultured parasites and one of these compounds, when administered parenterally, cured *Plasmodium vinckei*-infected mice. We now report an evaluation of orally administered peptidyl inhibitors of falcipain in a mouse malaria model. In studies with a fluoromethyl ketone, orally administered morpholine urea-phenylalanine-homophenylalanine-fluoromethyl ketone delayed the progression of murine malaria. In studies of a new series of vinyl sulfones, a set of related compounds demonstrated marked inhibition of falcipain and of parasite biological activities in vitro. One of these compounds, *N*-methyl piperazine urea-leucine-homophenylalanine-2-naphthalene vinyl sulfone, cured about 40% of mice when administered orally twice-a-day for four days. Our results suggest that peptidyl inhibitors of falcipain have promise as antimalarial chemotherapeutic agents.

Introduction

Malaria is one of the most important infectious diseases in the world. Infection with *Plasmodium falciparum*, the most virulent human malaria parasite, is responsible for hundreds of millions of illnesses and over one million deaths per year.[1] The control of malaria is complicated by the increasing resistance of malaria parasites to available drugs.[2] There is therefore a great need to develop new antimalarial agents. Among potential new targets for antimalarial drugs is the *P. falciparum* hemoglobinase falcipain.[3] Erythrocytic malaria parasites degrade hemoglobin to acquire amino acids for parasite protein synthesis.[4,5] Falcipain is a papain-family cysteine protease that appears to act in concert with other enzymes, including two aspartic proteases,[6,7] to degrade hemoglobin.[4,5] Incubation of erythrocytic parasites with inhibitors of falcipain blocks hemoglobin degradation and parasite development.[3,8–10] We are currently studying falcipain inhibitors as potential antimalarial agents.

Peptide-based falcipain inhibitors have demonstrated potent antimalarial effects. A number of compounds, including fluoromethyl ketones[8,11] and vinyl sulfones,[12] inhibited falcipain and blocked parasite hemoglobin degradation and development at nanomolar concentrations. In initial in vivo studies, when a fluoromethyl ketone was administered parenterally to *Plasmodium vinckei*-infected mice, parasite cysteine protease activity was blocked, and 80% of mice were cured.[11] We have now evaluated the in vivo efficacy of orally administered peptidyl inhibitors of falcipain. Fluoromethyl ketone and vinyl sulfone falcipain inhibitors demonstrated marked effects against murine malaria.

Chemistry

Morpholine urea-phenylalanine-homophenylalanine-fluoromethyl ketone (Mu-Phe-Hph-CH_2F) (Fig. 1) was synthesized by scientists at Prototek, Inc. by published methods.[13] Peptidyl vinyl sulfone protease inhibitors were synthesized from amino acid aldehydes using Wadsworth-Emmons chemistry, as previously described, by scientists at Arris Pharmaceutical (now Axys Pharmaceuticals).[14,15] Alteration in the parent compound morpholine urea-leucine-homophenylalanine-phenyl vinyl sulfone (Mu-Leu-HphVSPh) offered improved aqueous solubility with the replacement of the morpholine urea group with *N*-methyl piperazine urea (*N*-Me-pipu-Leu-HphVSPh) (Fig. 1). Secondly, the phenyl vinyl sulfone group was replaced with a naphthalene vinyl sulfone (*N*-Me-pipu-Leu-HphVS-2Np).

Results

Evaluation of in vitro antimalarial effects of peptidyl cysteine protease inhibitors

A number of peptidyl fluoromethyl ketone and vinyl sulfone cysteine protease inhibitors have been shown to

Key words: Malaria; *Plasmodium*; protease; proteinase; chemotherapy; cysteine protease; protease inhibitor.
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0968-0896/99/$ - see front matter
PII: S0968-0896(99)00004-8

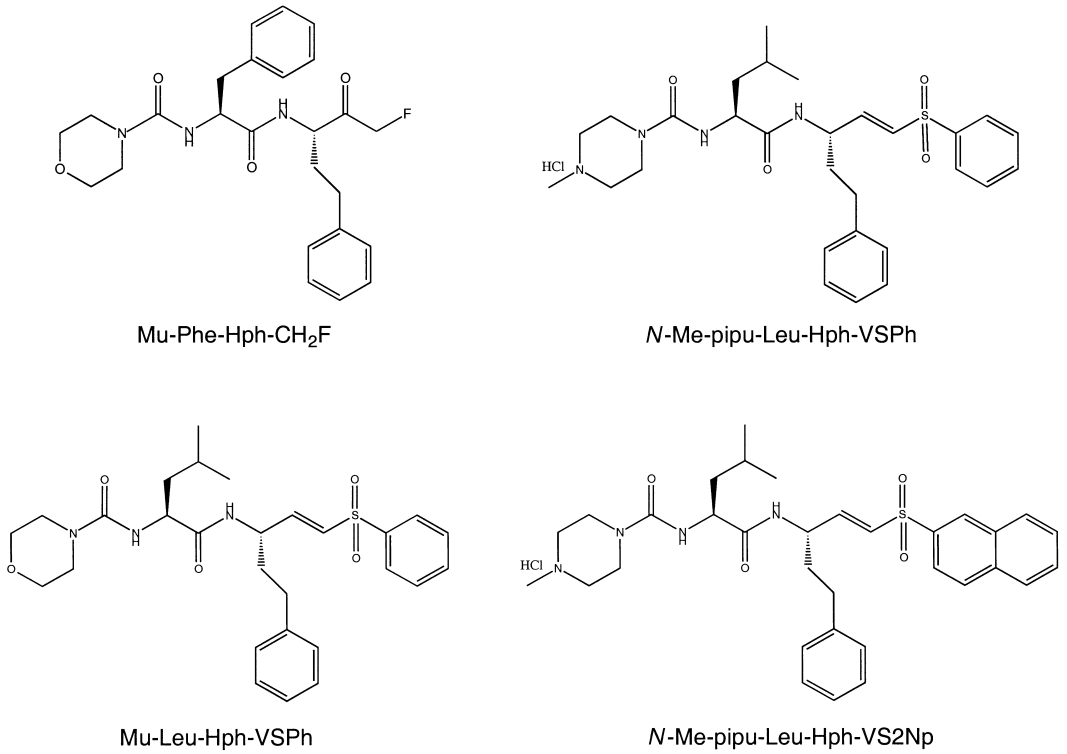

Figure 1. Structures of the peptidyl cysteine protease inhibitors discussed in this report.

be effective inhibitors of falcipain and to block parasite hemoglobin degradation and development at nanomolar concentrations.[8,11,12] The most promising fluoromethyl ketone in earlier studies was Mu-Phe-Hph-CH_2F, which strongly inhibited falcipain and, when administered parenterally, cured murine malaria.[11] The optimal vinyl sulfone was Mu-Leu-HphVSPh, which blocked hemoglobin degradation and development in cultured *P. falciparum* parasites at low nanomolar concentrations.[12]

To improve in vivo effectiveness, two analogues of Mu-Leu-HphVSPh were synthesized. In these compounds (*N*-Me-pipu-Leu-HphVSPh and *N*-Me-pipu-Leu-HphVS-2Np) the morpholine urea group was replaced with *N*-methyl piperazine urea to improve aqueous solubility, and presumably bioavailability. In addition, in one of the compounds, the phenyl vinyl sulfone group was replaced with a naphthalene vinyl sulfone. The peptidyl vinyl sulfones were potent inhibitors of falcipain (Table 1). The compounds also strongly inhibited hemoglobin degradation, development, and metabolic activity in cultured *P. falciparum* parasites (Table 2). Antimalarial effects correlated with potency against falcipain, as *N*-Me-pipu-Leu-HphVS-2Np was in each case the most effective inhibitor tested.

Evaluation of the in vivo antimalarial efficacy of a peptidyl fluoromethyl ketone inhibitor of falcipain

The peptidyl fluoromethyl ketone Mu-Phe-Hph-CH_2F was previously shown to be a potent inhibitor of falcipain and the *P. vinckei* analogue of falcipain[11] (Table 1). The compound also blocked parasite development at low nanomolar concentrations (Table 2). In initial in vivo studies, Mu-Phe-Hph-CH_2F effectively controlled murine malaria infections, although maximal efficacy required frequent (four doses/day) dosing.[11] To evaluate the efficacy of orally administered Mu-Phe-Hph-CH_2F as a chemoprophylactic drug, we supplied the compound with feed to BALB/c mice that were challenged with *P. vinckei* infection one day after the initiation of therapy. Animals that were treated with Mu-Phe-Hph-CH_2F at doses of 100 or 200 mg/kg/day demonstrated antimalarial effects, but not full protection. These animals developed malaria, which was eventually lethal, but the disease progressed more slowly in treated animals than in controls (Fig. 2). A dose response was seen, as the higher dose had a considerably greater effect. Higher doses of 300–400 mg/kg/day showed strong antimalarial efficacy, but these doses were not well tolerated beyond about three days, with the development of lethargy and anorexia, such that the higher doses were not reliably ingested. At the 100–200 mg/kg/day dosing, some animals were lethargic during treatment, but no severe toxicity was observed.

Table 1. Inhibition of plasmodial cysteine proteases by peptidyl cysteine protease inhibitors

Inhibitor	Inhibitory IC_{50} (nM)[a]	
	P. falciparum	*P. vinckei*
Mu-Phe-Hph-CH_2F	3[b]	5[b]
Mu-Leu-HphVSPh	10	300
N-Me-pipu-Leu-HphVSPh	5	200
N-Me-pipu-Leu-HphVS-2Np	2	20

[a]All assays were performed in an identical manner with a 30 nM concentration of either falcipain or the *P. vinckei* falcipain analogue.
[b]Results are from a prior study.[11]

Table 2. Inhibition of cultured *P. falciparum* parasites by peptidyl falcipain inhibitors

Compound	Inhibition of			
	Development[a] (nM)	Metabolism[b] (nM)	Hemoglobin degradation[c]	
			10 nM	1 nM
Mu-Phe-HPh-CH_2F	4	ND[d]	++	+
Mu-Leu-HphVSPh	4	200	++	–
N-Me-pipu-Leu-HphVSPh	2	100	+++	+
N-Me-pipu-Leu-HphVS-2Np	0.4	20	+++	++

[a]IC_{50}s for the inhibition of development, measured by counting new rings after a 48 h incubation.
[b]IC_{50}s for the inhibition of [^{3}H]hypoxanthine uptake.
[c]Food vacuole abnormality at two inhibitor concentrations, scored as discussed in Experimental.
[d]Not done.

Evaluation of the in vivo antimalarial efficacy of peptidyl vinyl sulfone inhibitors of falcipain

To evaluate the antimalarial efficacy of orally administered vinyl sulfones, we utilized a model for treatment of murine malaria. Swiss Webster mice were infected with 10^5 *P. vinckei*-infected erythrocytes, and treatment was initiated three days after infection. Animals treated with *N*-Me-pipu-Leu-HphVS-2Np twice a day by gastric lavage showed marked antimalarial effects. About 40% of animals treated with 50 or 100 mg/kg twice-a-day were cured of their infections, and the remainder of treated animals showed a markedly delayed progression of malaria, as compared to controls (Fig. 3). *N*-Me-pipu-Leu-HphVSPh was a less effective antimalarial. Animals treated with this compound twice-a-day were not cured, but as was the case with *N*-Me-pipu-Leu-HphVS-2Np, they showed a markedly delayed progression of their infections. In an experiment following the same protocol as that shown in Figure 3, animals treated with 100 mg/kg of *N*-Me-pipu-Leu-HphVSPh twice-a-day survived for a mean of 12.8 days after infection, as compared to untreated controls, who survived for 8.3 days. Some animals were lethargic during therapy with vinyl sulfones, but none demonstrated major toxicity.

Discussion

We have shown that orally administered peptidyl inhibitors of the *P. falciparum* cysteine protease falcipain had potent in vivo antimalarial effects in a murine malaria model. The fluoromethyl ketone Mu-Phe-Hph-CH_2F, which previously was shown to be effective in vivo when administered parenterally, was also effective in delaying the progression of murine malaria when administered orally. A new series of peptidyl vinyl sulfone falcipain inhibitors demonstrated strong inhibition of hemoglobin degradation and development in

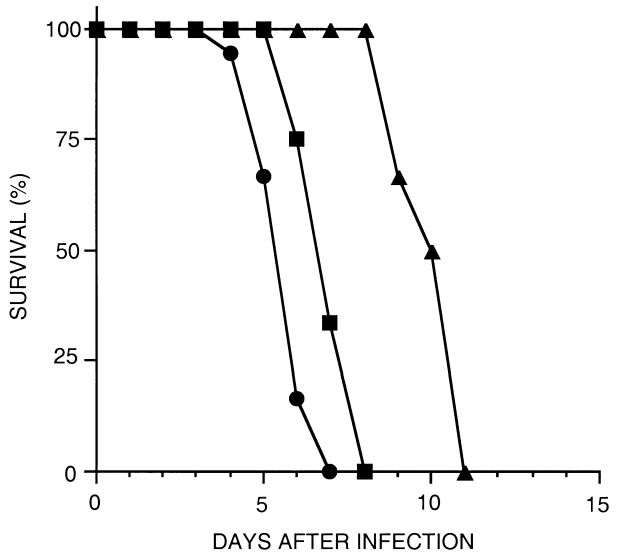

Figure 2. In vivo antimalarial effects of an orally administered fluoromethyl ketone. Mice were begun on therapy with Mu-Phe-Hph-CH_2F, and one day later infected with *P. vinckei*. Therapy was continued for 7 days after the initiation of infection, and parasitemias were evaluated daily. Mice were considered nonsurvivors when parasitemias topped 50%. Survival data for untreated control mice (circles, $n=18$) and animals treated with 100 mg/kg/day (squares, $n=12$) and 200 mg/kg/day (triangles, $n=6$) are shown.

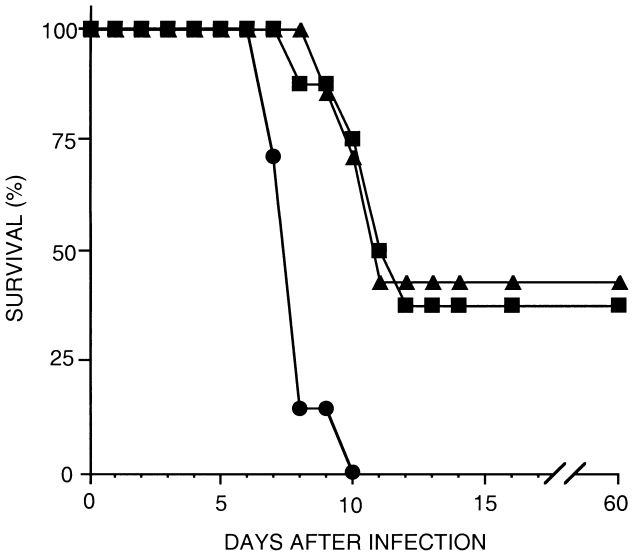

Figure 3. In vivo antimalarial effects of an orally administered vinyl sulfone. Mice were infected with *P. vinckei* and three days later begun on therapy with *N*-Me-pipu-Leu-HphVS-2Np, which was continued for 4 days. Parasitemias were evaluated daily, and mice were considered nonsurvivors when parasitemias topped 50%. Survival data for untreated control mice (circles, $n=7$) and animals treated with 100 mg/kg/day (squares, $n=8$), and 200 mg/kg/day (triangles, $n=7$) are shown.

cultured malaria parasites. When one of these compounds, *N*-Me-pipu-Leu-HphVS-2Np, was administered orally to mice, it markedly delayed the progression of murine malaria, and cured about 40% of treated animals.

The clinical efficacy of peptidyl protease inhibitors is potentially problematic, due to limited bioavailability, poor pharmacokinetics, and/or the inhibition of host proteases. However, peptide-based inhibitors of other proteases, including renin,[16] thrombin,[17] and leukocyte elastase,[18,19] have been shown to be orally bioavailable and, in a number of cases, biologically active after oral administration. For example, a peptide-based renin inhibitor that is somewhat larger than the compounds studied in this report was shown to have good oral bioavailability and in vivo activity in a number of species of animals.[16] Interestingly, in this case the primary limiting factor for bioavailability was not stability or absorption after oral administration, but rather hepatic elimination. In the case of the human immunodeficiency virus protease, numerous peptide-based, orally bioavailable compounds have been identified,[20–23] and some of these compounds are already in clinical use.[24]

Recent success with orally administered peptide-based compounds is encouraging, as oral effectiveness would add greatly to the utility of a new antimalarial. In addition, in our studies to date, peptidyl falcipain inhibitors have shown greater efficacy than nonpeptides. We are therefore attempting to optimize peptidyl falcipain inhibitors as orally effective antimalarials. Initial results with the fluoromethyl ketone Mu-Phe-Hph-CH_2F in therapy were promising,[11] and we now demonstrate that this compound has moderate efficacy when administered orally. However, the efficacy of fluoromethyl ketones was probably limited by poor solubility in vivo, and this class of compounds is also now known to be toxic in some animal systems. We therefore have more recently concentrated on studies of vinyl sulfones.

Prior studies[12] and this report highlight informative structure–activity relationships among peptidyl falcipain inhibitors. Considering amino acid sequences, studies with both fluoromethyl ketones[11] and vinyl sulfones[12] (Table 1) have identified Leu–Hph peptides as excellent inhibitors of falcipain. Interestingly, the *P. vinckei* analogue of falcipain differs, in that it is about as sensitive as falcipain to Phe–Hph peptides, but it is much less sensitive than is falcipain to Leu–Hph peptides.[11,12] Thus, while Leu–Hph compounds may be optimally effective against human *P. falciparum* malaria, their efficacy is expected to be understated in the *P. vinckei* animal model. Mu-Leu-HphVSPh also inhibited the human papain-family proteases cathepsins B, L, S, and O2 (K), although the activity of different peptides against these similar proteases differed markedly.[14,15] Precise comparisons of the inhibition of these enzymes by peptide inhibitors will aid in the design of selective inhibitors. A protocol for the affinity purification of falcipain has recently been developed (unpublished) and detailed comparisons of the inhibition of purified falcipain and host enzymes by vinyl sulfone inhibitors are planned.

Considering the amino-terminus of falcipain inhibitors, benzyloxycarbonyl, morpholine urea, and *N*-methylpiperazine urea groups are all acceptable in terms of enzyme inhibition.[8,11,12] The increased aqueous solubility afforded by the morpholine urea and *N*-methyl piperazine urea groups should improve in vivo bioavailability; we have concentrated on *N*-methyl piperazine urea-containing compounds in our recent in vivo studies.

Considering the active group of the peptide inhibitors, the vinyl sulfones appear to be less potent in inhibiting falcipain than are fluoromethyl ketones,[8,11,12] but optimal vinyl sulfone inhibitors are nonetheless very potent, and the vinyl sulfones do not appear to have the toxicity concerns of the fluoromethyl ketones. We previously showed that phenyl vinyl sulfones are much more potent than methyl vinyl sulfones.[12] In this report we have shown that the naphthalene vinyl sulfone group offers improved activity, compared to the phenyl vinyl sulfone, both in terms of the inhibition of falcipain and the inhibition of biological activities of cultured parasites. This result may relate to improved binding of the naphthalene-containing inhibitor to the 'primed' side of the falcipain active site; binding to both the 'primed' and 'unprimed' sides of the active site should improve inhibitor potency and selectivity.[25]

Upon in vivo evaluation, *N*-Me-pipu-Leu-HphVS-2Np markedly delayed the progression of murine malaria and cured about 40% of mice treated twice-a-day for four days. The compound was more effective than the equivalent phenyl vinyl sulfone. We do not yet have pharmacokinetic information on the half-life of *N*-Me-pipu-Leu-HphVS-2Np, though preliminary results with a related vinyl sulfone demonstrated a half-life of about 30 min in mice and rats (J. H. McKerrow, L. Z. Benet, Y. Zhang, J. C. Engel, personal communication). This result suggests that improved antimalarial efficacy with *N*-Me-pipu-Leu-HphVS-2Np should be seen with more frequent dosing. Alternatively, chemical modifications should improve the bioavailability and pharmacokinetics of falcipain inhibitors to allow infrequent oral administration for the prevention and treatment of malaria. Our present results suggest that peptidyl inhibitors of falcipain are appropriate lead compounds for antimalarial drug development.

Experimental

Parasites

It strain *P. falciparum* parasites were cultured with human erythrocytes at 2% hematocrit in RPMI media and either 10% human serum or Albumax I serum substitute as previously described.[12] Parasite synchrony was maintained with serial treatments of cultures with 5% D-sorbitol.[26] Frozen stocks of *P. vinckei* were used to infect BALB/c or Swiss Webster mice by ip injection. Parasites were subsequently passaged in mice by the ip injection of 10^5–10^6 *P. vinckei*-infected erythrocytes. Parasites from cultures or mice were evaluated on Giemsa-stained smears.

Assays of enzyme inhibition

Assays of the hydrolysis of the fluorogenic substrate benzyloxycarbonyl-phenylalanine-arginine-amino-4-methyl-coumarin (Z-Phe-Arg-AMC) were performed in a microplate format as previously described.[12] *P. falciparum* trophozoite extracts containing falcipain and *P. vinckei* extracts containing the falcipain analogue were prepared as previously described.[11] Equal concentrations of protease (30 nM, as determined with the stoichiometric cysteine protease inhibitor L-transepoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64))[27] were used in each assay. Fluoromethyl ketone and vinyl sulfone inhibitors were added to reactions (in 0.1 M sodium acetate and 10 mM dithiothreitol, pH 5.5, room temperature) from 100× stocks in DMSO 30 min before the substrate was added. Inhibitor concentrations were studied at least in duplicate at multiple concentrations in three separate experiments. The rate of hydrolysis of Z-Phe-Arg-AMC (increase in fluorescence/time, monitored continuously over 30 min) in the presence of the protease inhibitors was compared with the rate of hydrolysis in negative controls incubated with an equivalent volume of DMSO, and the concentrations yielding 50% inhibition were extrapolated from plots of mean percent control activity over inhibitor concentration.

Assays of hemoglobin degradation

To assess the effects of inhibitors on the accumulation of hemoglobin in *P. falciparum* trophozoites, synchronized ring-stage parasites were incubated at 37° in microtiter plate cultures with different concentrations of inhibitors added from 100× stocks in DMSO. After 24 h of incubation, Giemsa-stained smears were made from the parasite cultures, and parasites were evaluated for the presence of the dark-staining, swollen food vacuole that has previously been correlated with a block in hemoglobin degradation.[3,9] The food vacuole abnormality was scored as +++ (marked food vacuole swelling in nearly all parasites, with minimal development beyond the trophozoite stage), ++ (less marked abnormality in nearly all parasites), + (mild food vacuole abnormality, with continued development in many parasites), and − (no appreciable abnormality).

Assays of parasite metabolic activity and development

To assess the effects of inhibitors on the metabolic activity of cultured *P. falciparum* parasites, we utilized a modification of a standard assay of the uptake of [^{3}H]hypoxanthine,[28] as previously described.[12] To assess the effects of inhibitors on the development of cultured *P. falciparum* parasites, cultures were incubated with protease inhibitors (from 100× stocks in DMSO) 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 from the cultures, new ring forms per 1000 erythrocytes were counted, and the counts were compared with those from control parasites cultured in the equivalent (1%) concentration of DMSO. For both assays, concentrations yielding 50% inhibition were extrapolated from plots of mean percent control activity over inhibitor concentration.

Evaluation of the in vivo antimalarial effects of protease inhibitors

In evaluations of the fluoromethyl ketone protease inhibitor Mu-Phe-Hph-CH_2F, a model for chemoprophylaxis against malaria was utilized. BALB/c mice were begun on therapy by the inclusion of finely ground inhibitor in feed. Available feed was limited to 3 g per mouse per day, such that all feed was ingested each day, and inhibitor ingestion per cage could be quantitated. However, it was not possible to determine the individual doses ingested by each of 6 mice in each cage. Control animals were fed an equal amount without the protease inhibitor. Mice were infected with 10^6 *P. vinckei*-infected erythrocytes by ip injection 1 day after therapy with Mu-Phe-Hph-CH_2F was initiated, and therapy was continued for seven days after the initiation of infection. In evaluations of vinyl sulfone protease inhibitors, a model for the treatment of malaria was used. Swiss Webster mice were infected with 10^5 *P. vinckei*-infected erythrocytes by the ip injection of parasites from a previously-infected mouse. Three days later treatment was initiated with vinyl sulfones, which were administered by gastric lavage (50–100 μl in peanut oil) every 12 h for 4 days. Control animals received an equal volume of peanut oil without inhibitor. For all murine malaria experiments, mice were evaluated daily for toxicity and for parasitemia by evaluation of Giemsa-stained blood smears. Animals were sacrificed when severe toxicity was identified or when parasitemias topped 50%.

Acknowledgements

The authors thank Dr. Robert Smith, Prototek, Inc. for providing fluoromethyl ketones, Dr. James Palmer, Axys Pharmaceuticals, for providing vinyl sulfones, and Dr. James McKerrow, University of California, San Francisco, for a critical review of this manuscript. This work was supported by grants from the National Institutes of Health, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and the American Heart Association. P.J.R. is an Established Investigator of the American Heart Association.

References and Notes

1. Walsh, J. A. *Ann. N.Y. Acad. Sci.* **1989**, *569*, 1–16.
2. Olliaro, P.; Cattani, J.; Wirth, D. *JAMA* **1996**, *275*, 230–233.
3. Rosenthal, P. J.; McKerrow, J. H.; Aikawa, M.; Nagasawa, H.; Leech, J. H. *J. Clin. Invest*. **1988**, *82*, 1560–1566.
4. Rosenthal, P. J.; Meshnick, S. R. *Mol. Biochem. Parasitol.* **1996**, *83*, 131–139.
5. Francis, S. E.; Sullivan, D. J.; Goldberg, D. E. *Annu. Rev. Micro*. **1997**, *51*, 97–123.
6. Goldberg, D. E.; Slater, A. F. G.; Beavis, R.; Chait, B.; Cerami, A.; Henderson, G. B. *J. Exp. Med.* **1991**, *173*, 961–969.

7. Gluzman, I. Y.; Francis, S. E.; Oksman, A.; Smith, C. E.; Duffin, K. L.; Goldberg, D. E. *J. Clin. Invest*. **1994**, *93*, 1602–1608.
8. Rosenthal, P. J.; Wollish, W. S.; Palmer, J. T.; Rasnick, D. *J. Clin. Invest*. **1991**, *88*, 1467–1472.
9. Rosenthal, P. J. *Exp. Parasitol*. **1995**, *80*, 272–281.
10. Gamboa de Domínguez, N. D.; Rosenthal, P. J. *Blood* **1996**, *87*, 4448–4454.
11. Rosenthal, P. J.; Lee, G. K.; Smith, R. E. *J. Clin. Invest*. **1993**, *91*, 1052–1056.
12. Rosenthal, P. J.; Olson, J. E.; Lee, G. K.; Palmer, J. T.; Klaus, J. L.; Rasnick, D. *Antimicrob. Agents Chemother*. **1996**, *40*, 1600–1603.
13. Rasnick, D. *Anal. Biochem*. **1985**, *149*, 461–465.
14. Palmer, J. T.; Rasnick, D.; Klaus, J. L.; Brömme, D. *J. Med. Chem*. **1995**, *38*, 3193–3196.
15. Brömme, D.; Klaus, J. L.; Okamoto, K.; Rasnick, D.; Palmer, J. T. *Biochem. J*. **1996**, *315*, 85–89.
16. Kleinert, H. D.; Rosenberg, S. H.; Baker, W. R.; Stein, H. H.; Klinghofer, V.; Barlow, J.; Spina, K.; Polakowski, J.; Kovar, P.; Cohen, J.; Denissen, J. *Science* **1992**, *257*, 1940–1943.
17. Feng, D. M.; Gardell, S. J.; Lewis, S. D.; Bock, M. G.; Chen, Z.; Freidinger, R. M.; Naylor-Olsen, A. M.; Ramjit, H. G.; Woltmann, R.; Baskin, E. P.; Lynch, J. J.; Lucas, R.; Shafer, J. A.; Dancheck, K. B.; Chen, I. W.; Mao, S. S.; Krueger, J. A.; Hare, T. R.; Mulichak, A. M.; Vacca, J. P. *J. Med. Chem*. **1997**, *40*, 3726–3733.
18. Edwards, P. D.; Andisik, D. W.; Bryant, C. A.; Ewing, B.; Gomes, B.; Lewis, J. J.; Rakiewicz, D.; Steelman, G.; Strimpler, A.; Trainor, D. A.; Tuthill, P. A.; Mauger, R. C.; Veale, C. A.; Wildonger, R. A.; Williams, J. C.; Wolanin, D. J.; Zottola, M. *J. Med. Chem*. **1997**, *40*, 1876–1885.
19. Veale, C. A.; Bernstein, P. R.; Bohnert, C. M.; Brown, F. J.; Bryant, C.; Damewood, Jr. J. R.; Earley, R.; Feeney, S. W.; Edwards, P. D.; Gomes, B.; Hulsizer, J. M.; Kosmider, B. J.; Krell, R. D.; Moore, G.; Salcedo, T. W.; Shaw, A.; Silberstein, D. S.; Steelman, G. B.; Stein, M.; Strimpler, A.; Thomas, R. M.; Vacek, E. P.; Williams, J. C.; Wolanin, D. J.; Woolson, S. *J. Med. Chem*. **1997**, *40*, 3173–3181.
20. Abdel-Meguid, S. S.; Metcalf, B. W.; Carr, T. J.; Demarsh, P.; DesJarlais, R. L.; Fisher, S.; Green, D. W.; Ivanoff, L.; Lambert, D. M.; Murthy, K. H. M.; Petteway, S. R.; Pitts, W. J.; Tomaszek, T. A.; Winborne, E.; Zhao, B.; Dreyer, G. B.; Meek, T. D. *Biochemistry* **1994**, *33*, 11671–11677.
21. Kempf, D. J.; Marsh, K. C.; Denissen, J. F.; McDonald, E.; Vasavanonda, S.; Flentge, C. A.; Green, B. E.; Fino, L.; Park, C. H.; Kong, X. P.; Wideburg, N. E.; Saldivar, A.; Ruiz, L.; Kati, W. M.; Sham, H. L.; Robins, T.; Stewart, K. D.; Hsu, A.; Plattner, J. J.; Leonard, J. M.; Norbeck, D. W. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2484–2488.
22. Fässler, A.; Bold, G.; Capraro, H. G.; Cozens, R.; Mestan, J.; Poncioni, B.; Rosel, J.; Tintelnot-Blomley, M.; Lang, M. *J. Med. Chem*. **1996**, *39*, 3203–3216.
23. Smith, A. B.; Hirschmann, R.; Pasternak, A.; Yao, W.; Sprengeler, P. A.; Holloway, M. K.; Kuo, L. C.; Chen, Z.; Darke, P. L.; Schleif, W. A. *J. Med. Chem*. **1997**, *40*, 2440–2444.
24. Deeks, S. G.; Smith, M.; Holodniy, M.; Kahn, J. O. *JAMA* **1997**, *277*, 145–153.
25. Thompson, S. K.; Halbert, S. M.; Bossard, M. J.; Tomaszek, T. A.; Levy, M. A.; Zhao, B.; Smith, W. W.; Abdel-Meguid, S. S.; Janson, C. A.; D'Alessio, K. J.; McQueney, M. S.; Amegadzie, B. Y.; Hanning, C. R.; DesJarlais, R. L.; Briand, J.; Sarkar, S. K.; Huddleston, M. J.; Ijames, C. F.; Carr, S. A.; Garnes, K. T.; Shu, A.; Heys, J. R.; Bradbeer, J.; Zembryki, D.; Lee-Rykaczewski, L.; James, I. E.; Lark, M. W.; Drake, F. H.; Gowen, M.; Gleason, J. G.; Veber, D. F. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 14249–14254.
26. Lambros, C.; Vanderberg, J. P. *J. Parasitol*. **1979**, *65*, 418–420.
27. Barrett, A. J.; Kirschke, H. *Meth. Enzymol*. **1981**, *80*, 535–561.
28. Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. *Antimicrob. Agents Chemother*. **1979**, *16*, 710–718.