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Antimalarial Effects of Vinyl Sulfone Cysteine Proteinase Inhibitors

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We evaluated the antimalarial effects of vinyl sulfone cysteine proteinase inhibitors. A number of vinyl sulfones strongly inhibited falcipain, a *Plasmodium falciparum* cysteine proteinase that is a critical hemoglobinase. In studies of cultured parasites, nanomolar concentrations of three vinyl sulfones inhibited parasite hemoglobin degradation, metabolic activity, and development. The antimalarial effects correlated with the inhibition of falcipain. Our results suggest that vinyl sulfones or related cysteine proteinase inhibitors may have promise as antimalarial agents.

Malaria is one of the most important infectious diseases in the world, causing more than 100 million cases of illness and 1 million deaths per year (18). A major factor in the persistence of widespread malaria is the increasing resistance of malaria parasites, in particular, *Plasmodium falciparum*, to available drugs (7). There is therefore a great need to identify new targets for antimalarial chemotherapy and to develop new drugs directed against these targets.

Potential targets for chemotherapy include enzymes involved in the degradation of hemoglobin by intraerythrocytic malaria parasites. These parasites degrade hemoglobin in an acidic food vacuole to provide free amino acids for parasite protein synthesis. Malarial parasite cysteine and aspartic-class proteinases have been identified, localized to the food vacuole, and shown to degrade hemoglobin in vitro (3, 4, 13, 16, 17). Inhibitors of the cysteine proteinase falcipain have been shown to block the hydrolysis of globin (11, 13, 15) and to inhibit the processing of native hemoglobin (2) by cultured parasites. Specific inhibitors of falcipain have been studied as potential antimalarial agents. Peptidyl fluoromethyl ketones were potent inhibitors of falcipain and blocked hemoglobin degradation and parasite development when they were incubated with cultured parasites at nanomolar concentrations (15). One of these compounds, morpholine urea (Mu)-Phe-homophenylalanine (HPh)-CH_2F, cured 80% of malaria-infected mice when it was administered subcutaneously over 4 days (12). Nonpeptide inhibitors of falcipain developed through a molecular modeling approach also inhibited the metabolic activities of cultured parasites (6, 10). However, further development of fluoromethyl ketones as drugs is not planned because of the toxicities of these compounds in experimental animals, and the potencies of nonpeptide inhibitors have not yet approached those achieved with peptides.

In an attempt to identify nontoxic peptide-based cysteine proteinase inhibitors, a series of vinyl sulfones was recently synthesized and evaluated (9). A number of compounds were potent cysteine proteinase inhibitors. We now present data from a study in which we evaluated these vinyl sulfones as antimalarial agents. Potent inhibitors of falcipain were identified and were shown to exhibit strong antimalarial effects in vitro. The results suggest that vinyl sulfones or related compounds directed against falcipain may have promise as antimalarial drugs.

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MATERIALS AND METHODS

Parasites. Itg2 strain *P. falciparum* parasites were cultured with human erythrocytes at 2% hematocrit in RPMI medium with either 10% human serum or serum substitute (final concentrations of 0.5% AlbuMAX I, 1% 100× HT (10 mM sodium hypoxanthine, 1.6 mM thymidine) supplement, and 1% minimum essential medium–100× sodium pyruvate solution; all from Gibco BRL). Synchrony was maintained with sorbitol (5). Frozen stocks of *Plasmodium vinckei* (kindly provided by William Weidanz, University of Wisconsin) were used to infect BALB/c mice by intraperitoneal injection. Parasites were subsequently passaged in mice. Parasites from culture or mice were evaluated with Giemsa-stained smears.

Synthesis of vinyl sulfones. Peptidyl methyl and phenyl vinyl sulfones were synthesized from amino acid aldehydes by Wadsworth-Emmons chemistry as described recently (9). The potencies of the compounds against cysteine proteinases were demonstrated by using fluorogenic peptide substrates (9). The compounds were shown to be >99% pure by high-pressure liquid chromatography.

Assays of enzyme inhibition. Assays of the hydrolysis of the fluorogenic substrate benzyloxycarbonyl-Phe-Arg-7-amino-4-methyl-coumarin (Z-Phe-Arg-AMC) were performed as described previously (14) except that a 96-well format was used for spectrofluorometry. *P. falciparum* trophozoite extracts containing falcipain and *P. vinckei* extracts containing the analogous *P. vinckei* cysteine proteinase were prepared as described previously (12). For each of multiple experiments, extracts (in 0.1 M sodium acetate and 10 mM dithiothreitol [pH 5.5]) containing identical concentrations of enzyme [~30 nM; calculated by titration with the stoichiometric cysteine proteinase inhibitor L-transepoxy-succinyl-leucylamido-(4-guanidino)-butane (E-64; Sigma)] were incubated with each vinyl sulfone inhibitor (added from 100× stocks in dimethyl sulfoxide [DMSO]) for 30 min at room temperature before the substrate was added. Inhibitor concentrations, studied in duplicate or triplicate in each experiment, ranged from those that minimally inhibited the enzyme to those that fully inhibited the enzyme. Fluorescence caused by the cleavage of Z-Phe-Arg-AMC was monitored continuously over 30 min at room temperature with a Labsystems Fluoroskan II spectrofluorometer. The rate of hydrolysis of Z-Phe-Arg-AMC (increase in fluorescence/time) in the presence of the vinyl sulfones was compared with the rate of hydrolysis in negative controls incubated with an equivalent volume of DMSO and with positive controls incubated with E-64 (10 μM).

Assays of hemoglobin degradation. To assess the effects of inhibitors on the accumulation of hemoglobin in trophozoites microscopically, synchronized ring-stage parasites were incubated at 37°C in microtiter plate cultures with vinyl sulfones added from 100× stocks in DMSO. Parasites were also incubated with the cysteine proteinase inhibitor E-64 (10 μM) as a control. After 24 h of incubation, Giemsa-stained smears were made from the parasite cultures, and the parasites were evaluated for the presence of the marked food vacuole abnormality that has previously been correlated with a block in hemoglobin degradation (11, 13). To assess hemoglobin accumulation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), parasites cultured with inhibitors as noted above were collected after 24 h, processed as described previously with saponin lysis of erythrocyte membranes and multiple washes (11), and

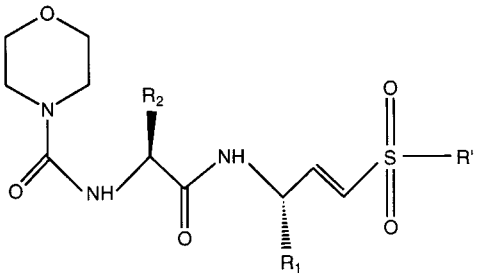

FIG. 1. Structures of the vinyl sulfones studied. All of the compounds contained an Mu-blocking group. R_1 and R_2 represent the side chains of the P1 and P2 amino acids, respectively, and R′ represents the methyl or phenyl substitutent.

electrophoresed in SDS–15% polyacrylamide gels. The gels were subsequently stained with Coomassie blue, and the accumulated globin was quantified by densitometry. Additionally, to evaluate membranes for hemoglobin association, saponin-lysed samples were subjected to hypotonic lysis by solubilization in H_2O; this was followed by two freeze-thaw cycles at −70°C. Membranes (and control unlysed parasites) were then collected by centrifugation (100,000 × *g* for 30 min at 4°C), washed in phosphate-buffered saline (PBS), and analyzed by SDS-PAGE.

Assays of parasite metabolic activity and development. To assess the effects of inhibitors on parasite metabolic activity, we used a modification of a standard [^{3}H]hypoxanthine uptake assay (1), as follows. Microwell cultures of ring-stage parasites were incubated with vinyl sulfones (from 100× stocks in DMSO) for 24 h, [^{3}H]hypoxanthine (Dupont-NEN) was added (0.0144 μg per well; 1.2 μCi per well), and the cultures were maintained for an additional 18 h. The cells were then harvested and deposited onto glass-fiber filters that were washed with water and dried with ethanol. [^{3}H]hypoxanthine uptake was quantitated by scintillation counting of the filters, and the uptake by treated cultures was compared with that by control cultures containing 1% DMSO. To assess the effects of inhibitors on parasite development, the cultures were incubated with vinyl sulfones (from 100× stocks in DMSO) for 48 h beginning at the ring stage. The 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 parasite cultures, the numbers of new ring forms per 1,000 erythrocytes were counted, and the counts were compared with those of controls cultured in 1% DMSO.

RESULTS

Inhibition of malarial cysteine proteinases by vinyl sulfones. Peptidyl vinyl sulfones (Fig. 1) were recently shown to be potent inhibitors of cysteine proteinases (9). We examined the abilities of vinyl sulfones to inhibit the *P. falciparum* food vacuole cysteine proteinase falcipain (13) and its analog in the murine malaria parasite *P. vinckei* (12). A number of phenyl vinyl sulfones were potent inhibitors of the malarial cysteine proteinases (Table 1). The effectiveness of the vinyl sulfones varied widely. For most compounds, inhibitory effects against falcipain and its analog in *P. vinckei* were similar. In some instances, however, effectiveness against the two related enzymes differed significantly, indicating the critical role of the peptide portion of the inhibitors in their specificity for these similar enzymes. Of interest, the compound Mu-Phe-Hph-VSPh (see footnote *a* of Table 1 for definitions of compound abbreviations) was a mid-nanomolar inhibitor of both malarial proteinases. The structurally similar peptide Mu-Leu-Hph-VSPh had markedly improved effectiveness against falcipain, while activity against the *P. vinckei* enzyme decreased. A similar pattern of inhibition was seen previously with the same two peptides bound to fluoromethyl ketone leaving groups (12).

Inhibition of malarial hemoglobin degradation by vinyl sulfones. Cultured *P. falciparum* parasites were incubated with three vinyl sulfones that were potent inhibitors of falcipain, and the effects of the vinyl sulfones on hemoglobin degradation were assessed morphologically and by SDS-PAGE analysis of parasite proteins. When parasites were incubated with the vinyl sulfones for 24 h beginning at the ring stage, the parasites developed swollen food vacuoles with the staining characteristics of erythrocyte cytoplasm. We have previously shown this specific abnormality to be indicative of a block in hemoglobin hydrolysis (11, 13). On the basis of these morphological criteria, hemoglobin degradation was markedly blocked at nanomolar concentrations of the three vinyl sulfones (Table 2). To confirm that the food vacuole abnormality caused by the vinyl sulfones was due to a block in hemoglobin degradation, we isolated parasite proteins after a 24-h incubation with the three compounds and evaluated the proteins by SDS-PAGE. By this assay, proteins from parasites incubated with nanomolar concentrations of the vinyl sulfones contained much more undegraded globin than did proteins from control parasites (Fig. 2;

TABLE 1. Inhibition of malarial cysteine proteinases by vinyl sulfones

Compound[a]	IC_{50} (μM)[b]	
	P. falciparum	*P. vinckei*
Mu-Phe-Ala-VSMe	20	ND[c]
Mu-Phe-Hph-VSMe	1	1
Mu-Phe-Phe-VSMe	2	ND
Mu-Phe-Arg-VSPh · HBr	0.05	0.05
Mu-Phe-Hph-VSPh	0.08	0.1
Mu-Phe-Lys(εZ)-VSPh	0.1	0.07
Mu-Phe-Lys-VSPh · HBr	0.09	0.05
Mu-Phe-Val-VSPh	1	1
Mu-Phe-Ser(OBzl)-VSPh	1	0.5
Mu-Leu-Hph-VSPh	0.003	0.2

[a] Standard amino acid abbreviations are used. Other abbreviations: Mu, morpholine urea; VS, vinyl sulfone; Me, methyl; Ph, phenyl; HBr, hydrogen bromide salt; Hph, homophenylalanine; Lys(εZ), benzyloxycarbonyl group bound to the lysine epsilon amino group; Ser(OBzl), benzyl group bound to the serine side chain hydroxyl group.

[b] The 50% inhibitory concentrations (IC_{50}s) were extrapolated from plots of the mean inhibition (4 to 12 assays per inhibitor) of enzyme activity by multiple concentrations of each inhibitor.

[c] ND, not done.

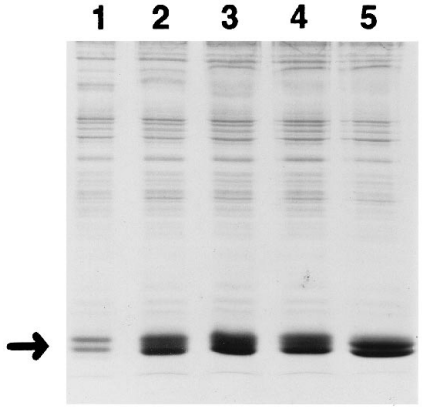

FIG. 2. Inhibition of hemoglobin degradation by vinyl sulfones. Cultured parasites were incubated with inhibitors for 24 h beginning at the ring stage, trophozoite-infected erythrocytes were collected and lysed with saponin, parasites were washed extensively, and parasite proteins were electrophoresed in SDS–15% polyacrylamide gels and then stained with Coomassie blue. The contents of approximately 10^7 parasites were included in each lane. Parasites incubated with the vinyl sulfones Mu-Phe-HPh-VSPh (lane 2; 0.3 μM), Mu-Phe-Lys(εZ)-VSPh (lane 3; 0.3 μM), and Mu-Leu-HPh-VSPh (lane 4; 0.03 μM) and with the cysteine proteinase inhibitor E-64 (lane 5; 10 μM) accumulated much more undegraded globin (arrow) than did controls incubated with DMSO (lane 1; 1%).

TABLE 2. Inhibition of cultured *P. falciparum* parasites by vinyl sulfones

Compound	Inhibitory concn (μM)			
	Hemoglobin degradation		Metabolism and development	
	Morphological abnormality in food vacuole[a]	Hemoglobin accumulation[b]	Hypoxanthine uptake[c]	Development in culture[d]
Mu-Phe-HPh-VSPh	0.3	0.1	2	0.2
Mu-Phe-Lys(εZ)-VSPh	0.3	0.1	3	0.2
Mu-Leu-HPh-VSPh	0.03	0.01	0.2	0.01

[a] Minimum concentrations that caused an abnormality in >50% of parasites after a 24-h incubation.
[b] Minimum concentrations that caused the accumulation of more than twice the control value of hemoglobin after a 24-h incubation, as determined by SDS-PAGE.
[c] The 50% inhibitory concentrations for the inhibition of [^{3}H]hypoxanthine uptake by cultured parasites.
[d] The 90% inhibitory concentrations for the inhibition of development, measured by counting new rings after a 48-h incubation.

Table 2). The apparent association of parasites and globin was not due simply to binding of hemoglobin to erythrocyte or parasite membranes, because electrophoresis of membranes isolated from inhibitor-treated parasites identified minimal globin (data not shown). As determined by both assays, vinyl sulfones inhibited malarial hemoglobin degradation in a dose-dependent manner.

Inhibition of parasite metabolic activity and development by vinyl sulfones. The effects of vinyl sulfones on parasite metabolic activity were assessed by a standard assay that measured the uptake of [^{3}H]hypoxanthine by cultured parasites. Parasite development was assessed by counting new ring-form parasites after control cultures had undergone a full developmental cycle. Parasite metabolic activity and development were strongly inhibited by the three vinyl sulfones that blocked hemoglobin degradation (Table 2). The assay for parasite development (counts of new ring forms) was considerably more sensitive than that for metabolic activity (uptake of [^{3}H]hypoxanthine). This result suggests that effective concentrations of the vinyl sulfones did not immediately kill the cultured parasites. Rather, the compounds likely starved the parasites for amino acids such that metabolic activity was gradually disrupted, and the subsequent completion of the developmental cycle of the parasites was prevented.

DISCUSSION

We have shown that vinyl sulfone cysteine proteinase inhibitors are potent inhibitors of falcipain, a *P. falciparum* cysteine proteinase that has been identified as a critical hemoglobinase and potential drug target. Three vinyl sulfones blocked falcipain activity, parasite hemoglobin degradation, and parasite development at nanomolar concentrations. Mu-Leu-Hph-VSPh, the vinyl sulfone that most effectively inhibited falcipain, also most effectively blocked hemoglobin degradation, [^{3}H]hypoxanthine uptake, and parasite development. A correlation between the potencies of compounds against falcipain and their effectiveness against parasite hemoglobin degradation, metabolic activity, and development was also seen earlier with fluoromethyl ketone inhibitors (15). These correlations strongly suggest that the antimalarial effects of the inhibitors were due to the specific inhibition of falcipain rather than some nonspecific antimalarial effect.

Ideally, antimalarial cysteine proteinase inhibitors will not inhibit host proteinases. Our results showing marked differences in the effects of some inhibitors against similar *P. falciparum* and *P. vinckei* proteinases suggest that careful selection of the peptide sequence can provide the desired specificity between host and parasite enzymes. However, some inhibition of host cysteine proteinases may be tolerable. The vinyl sulfones that we studied, which effectively inhibited host cysteine proteinases in vitro (9), have not shown measurable toxicity in extensive tests with animals. Oral administration of Mu-Phe-HPh-VSPh, Mu-Phe-Lys(εZ)-VSPh, or Mu-Leu-HPh-VSPh to rats at up to 30 mg/kg of body weight per day for 28 days caused no apparent toxicity or pathology (8). The fluoromethyl ketones studied earlier were somewhat toxic, but this appeared to be due to the generation of toxic metabolites and not host proteinase inhibition, because toxicity did not correlate with the inhibition of host enzymes. Thus, the current results suggest that cysteine proteinase inhibitors may be effective in the treatment of malaria and possibly other diseases even if the compounds somewhat inhibit host proteinases. In these cases host proteinases may not be accessible to the inhibitors and/or their full activity may not be required by the host, at least during short-term therapy. Study of the effects of vinyl sulfones against malaria parasites in vivo will be helpful in further evaluating their utility as antimalarial drugs.

Our data add to prior evidence suggesting that falcipain is a promising target for antimalarial chemotherapy. As with the fluoromethyl ketones studied previously, with each of the three vinyl sulfones studied in detail, effectiveness against falcipain in a simple fluorometric assay directly predicted antimalarial effectiveness. Our results with both fluoromethyl ketones and vinyl sulfones suggest that the peptide Mu-Leu-Hph may be an ideal inhibitor of falcipain and *P. falciparum* parasites. Alteration in blocking or leaving groups bound to this or related peptides may provide an optimally effective antimalarial agent.

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