ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2003, p. 154–160 Vol. 47, No. 1
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.1.154–160.2003

Structure-Activity Relationships for Inhibition of Cysteine Protease Activity and Development of *Plasmodium falciparum* by Peptidyl Vinyl Sulfones

Bhaskar R. Shenai,[1]* Belinda J. Lee,[1] Alejandro Alvarez-Hernandez,[2] Pek Y. Chong,[2] Cory D. Emal,[2] R. Jeffrey Neitz,[2] William R. Roush,[2] and Philip J. Rosenthal[1]

Department of Medicine, San Francisco General Hospital, University of California, San Francisco, California,[1] and Department of Chemistry, University of Michigan, Ann Arbor, Michigan[2]

Received 3 June 2002/Returned for modification 28 August 2002/Accepted 24 September 2002

The *Plasmodium falciparum* cysteine proteases falcipain-2 and falcipain-3 appear to be required for hemoglobin hydrolysis by intraerythrocytic malaria parasites. Previous studies showed that peptidyl vinyl sulfone inhibitors of falcipain-2 blocked the development of *P. falciparum* in culture and exerted antimalarial effects in vivo. We now report the structure-activity relationships for inhibition of falcipain-2, falcipain-3, and parasite development by 39 new vinyl sulfone, vinyl sulfonate ester, and vinyl sulfonamide cysteine protease inhibitors. Levels of inhibition of falcipain-2 and falcipain-3 were generally similar, and many potent compounds were identified. Optimal antimalarial compounds, which inhibited *P. falciparum* development at low nanomolar concentrations, were phenyl vinyl sulfones, vinyl sulfonate esters, and vinyl sulfonamides with P_2 leucine moieties. Our results identify independent structural correlates of falcipain inhibition and antiparasitic activity and suggest that peptidyl vinyl sulfones have promise as antimalarial agents.

Malaria is one of the most important infectious diseases in the world. *Plasmodium falciparum*, the most virulent human malaria parasite, is estimated to cause over 300 million new cases and 1 million deaths annually (33). Further complicating this grim scenario is the emergence of the widespread resistance of *P. falciparum* to available antimalarial drugs (25). New drugs to combat malaria are urgently needed.

Among potential new targets for antimalarial chemotherapy are enzymes that mediate hemoglobin hydrolysis. Intraerythrocytic *P. falciparum* trophozoites derive amino acids for protein synthesis from the hydrolysis of host cell hemoglobin in an acidic food vacuole (12, 20, 27). Proteases that hydrolyze hemoglobin in the food vacuole include members of the aspartic protease (1), cysteine protease (38, 39), and metalloprotease (9) families. Cysteine protease inhibitors arrested the erythrocytic life cycle of *P. falciparum* (26). Examination of inhibitor-treated parasites revealed abnormally swollen food vacuoles filled with undigested hemoglobin, indicating that the block in parasite development was due to the inhibition of hemoglobin hydrolysis (26).

P. falciparum contains three fairly typical papain family cysteine proteases, known as falcipains (28, 38, 39). Falcipain-2 and falcipain-3 appear to be the principal cysteine protease hemoglobinases (38, 39). Both of these proteases localize to vacuolar parasite fractions and readily hydrolyze hemoglobin under physiological reducing conditions at acidic pHs (37). Falcipain-2 is considerably more active against small peptide substrates, but the specificities of the two proteases are similar; both enzymes display a strong preference for leucine at the P_2 position (38, 39). The role of falcipain-1 in hemoglobin hydrolysis is unknown.

In earlier studies, peptidyl vinyl sulfones inhibited falcipain-2 activity and parasite development at nanomolar concentrations and were active in vivo against murine malaria (22, 29, 30). We have now initiated efforts to define structure-activity relationships (SAR) for the inhibition of falcipain-2, falcipain-3, and parasite development by a new series of peptidyl vinyl sulfones, vinyl sulfonate esters, and vinyl sulfonamides. We show that SAR for the two proteases are similar and that multiple compounds are potent inhibitors of the falcipains and of parasite development. However, the structural correlates for inhibition of the proteases differ notably from those for the inhibition of parasite development.

MATERIALS AND METHODS

Synthesis of vinyl sulfones, sulfonamides, and sulfonate esters. All inhibitors studied had a peptide backbone with multiple substituents at the P_3 position, Phe or Leu at P_2, and homoPhe or *O*-(phenyl)Ser at P_1; based on the substituents at the P_1' position, the inhibitors were further subclassified into phenyl vinyl sulfones, vinyl sulfonamides, or vinyl sulfonate esters (Fig. 1). These inhibitors were synthesized by using appropriate modifications of previously described methods (31, 32). The series of phenyl vinyl sulfones was prepared by a Horner-Wadsworth-Emmons reaction of *N-tert*-butoxycarbonyl (*N*-BOC)-homophenylalanal (Fig. 2, compound labeled 1) (23) with diethyl [(phenylsulfonyl)methyl]phosphonate (10) to produce *N*-BOC-homophenylalanyl phenyl vinyl sulfone (compound labeled 2). The BOC group was removed with trifluoroacetic acid, and the resulting amines were coupled to R_3 substituents under standard peptide coupling conditions (Fig. 2).

Vinyl sulfonamides [with homoPhe or *O*-(phenyl)Ser at P_1] and vinyl sulfonate esters were synthesized via the vinyl sulfonyl chlorides labeled 6 and 7 in Fig. 3 by using the general sequence reported by Gennari et al. (13) with appropriate modifications. Vinyl sulfonamides were synthesized by a Horner-Wadsworth-Emmons reaction of *N*-BOC-homophenylalanal (Fig. 3, structure 1) with triethyl-α-phosphorylmethanesulfonate (5, 21) to produce the *N*-BOC-homophenylalanyl vinyl sulfonate ethyl ester labeled 4 in Fig. 3. The sulfonate ester was dealkylated with tetrabutylammonium iodide and converted to the sulfonyl chloride labeled 6 in Fig. 3 with triphosgene and catalytic *N*-dimethylformamide (24).

* Corresponding author. Mailing address: Box 0811, University of California, San Francisco, San Francisco, CA 94143-0811. Phone: (415) 206-3352. Fax: (415) 648-8425. E-mail: bshenai@itsa.ucsf.edu.

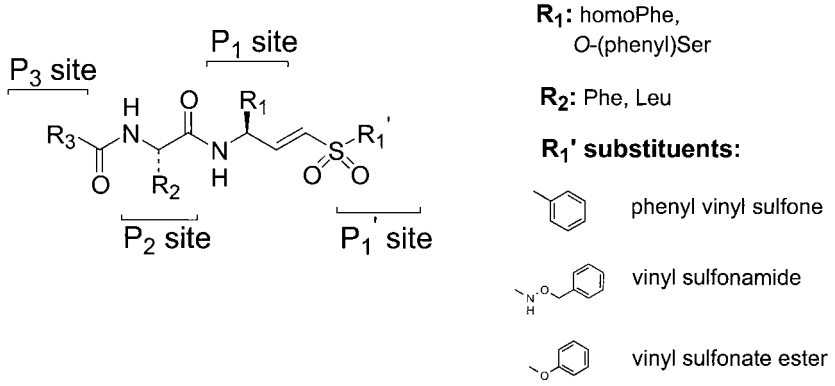

FIG. 1. General structures of the peptidyl vinyl sulfones studied. All compounds except 371 and 373 contained a urea linkage between the R_3 substituent and the peptide scaffold. R_1 and R_2 represent the side chains of the P_1 and the P_2 amino acids, respectively; R_1' represents vinyl sulfone, sulfonamide, or sulfonate ester substituents.

O-Benzylhydroxylamine was added to the sulfonyl chloride labeled 6 (Fig. 3) in the presence of 2,6-lutidine to form vinyl sulfonamide (compound labeled 8). The BOC group was removed with trifluoroacetic acid, and the resulting amine was coupled to R_3 substituents under standard peptide coupling conditions. A series of vinyl sulfonamides and vinyl sulfonate esters (with Leu at the P_2 and homoPhe at the P_1 position) was synthesized by analogous procedures via compounds 6 and 7 (Fig. 4 and 5). The *O*-phenyl serine derivative labeled 3 was synthesized by the general procedure of Cherney (7) with the appropriate modifications.

Assays of enzyme inhibition. IC_{50}s against falcipain-2 were determined as described earlier (30). Briefly, equal amounts (~1 nM) of recombinant falcipain-2 (38) were incubated with different concentrations of vinyl sulfones (added from 100× stocks in dimethyl sulfoxide [DMSO]) in 100 mM sodium acetate (pH 5.5)–10 mM dithiothreitol for 30 min at room temperature before addition of the substrate benzoxycarbonyl-Leu-Arg-7-amino-4-methyl-coumarin (final concentration, 25 μM). Fluorescence was continuously monitored for 30 min at room temperature in a Labsystems Fluoroskan II spectrofluorometer. IC_{50}s were determined from plots of activity over enzyme concentration with GraphPad Prism software.

For the determination of second-order binding constants, the concentrations of falcipain-2 and falcipain-3 were determined by active-site titration with benzoxycarbonyl-Phe-Arg-fluoromethyl ketone (39). Inhibitor assays were performed under pseudo-first-order conditions (i.e., the concentration of the inhibitor was at least 10-fold higher than that of the enzyme; substrate hydrolysis, <5%) by using the progress curve method (41). Falcipain-2 (0.6 nM) or falcipain-3 (0.8 to 1.2 nM) was incubated with different inhibitor concentrations in a solution containing 100 mM sodium acetate buffer (pH 5.5), 10 mM dithiothreitol, and the substrate benzoxycarbonyl-Leu-Arg-7-amino-4-methyl-coumarin (25 μM for falcipain-2 and 100 μM for falcipain-3). Product formation was continuously monitored in a Labsystems Fluoroskan II spectrofluorometer for 10 min at room temperature. To determine the observed first-order inactivation rate constant (k_{obs}), progress curves (fluorescence versus time) were analyzed by nonlinear-regression analysis (GraphPad Prism software) using the pseudo-first-

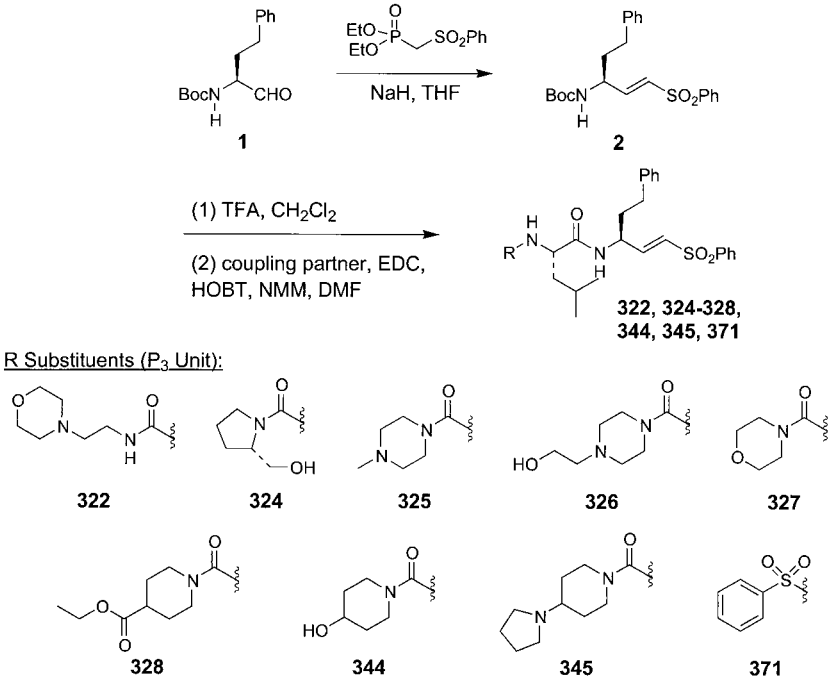

FIG. 2. Scheme for the synthesis of vinyl phenyl sulfones with Leu at the P_2 position and homoPhe at the P_1 position. Et, ethyl; Ph, phenyl; TFA, trifluoroacetic acid; THF, tetrahydofuran; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole hydrate; NMM, *N*-methylmorpholine; DMF, *N*-dimethylformamide.

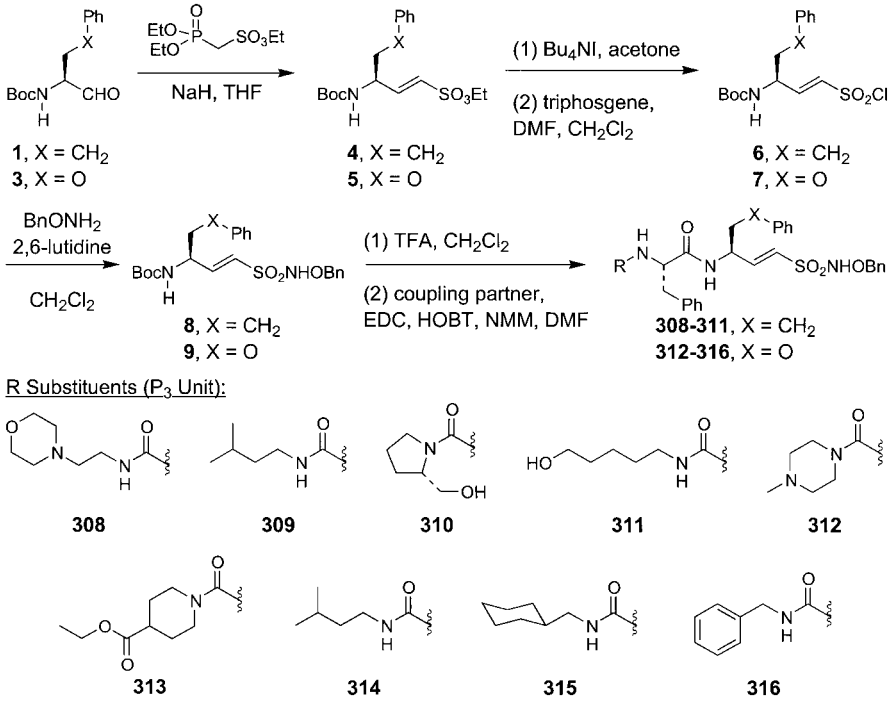

FIG. 3. Scheme for the synthesis of vinyl sulfonamides with Phe at the P_2 position and homoPhe or *O*-(phenyl)Ser at the P_1 position. Bn, benzyl; Bu, butyl. For all other abbreviations, see the legend to Fig. 2.

order rate equation $y = A \times (1 - e^{-k_{obs} \cdot t}) + B$, where y is the fluorescence at time t, A is the amplitude of the reaction, and B is the offset. Plots of k_{obs} versus the inhibitor concentration ([I]) were then used to determine uncorrected second-order rate constants (6), and for each inhibitor, the corrected second-order rate constant (k_{ass}, the association rate constant) was determined with the equation $k_{ass} = (k_{obs}/[I]) \times (1 + [S]/K_m)$ (4), where [S] is the substrate concentration.

Assay of parasite development. Effects of inhibitors on parasite development were determined as described earlier (30). Briefly, synchronized W2 strain *P. falciparum* parasites (18) were cultured with vinyl sulfones (added from 1,000× 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. Giemsa-stained smears were made after 48 h, when control cultures contained nearly all ring-stage parasites. The number of new ring forms per 500 erythrocytes was counted, and counts were compared with those of controls cultured in 0.1% DMSO. IC_{50}s for growth inhibition were determined with GraphPad Prism software from plots of percentages of the level of parasitemia of the control relative to inhibitor concentration.

RESULTS

Inhibition of falcipain-2 by vinyl sulfones. Our strategy was to build on initial results that demonstrated potent inhibition of falcipain-2 by peptidyl vinyl sulfones (30). Limited prior

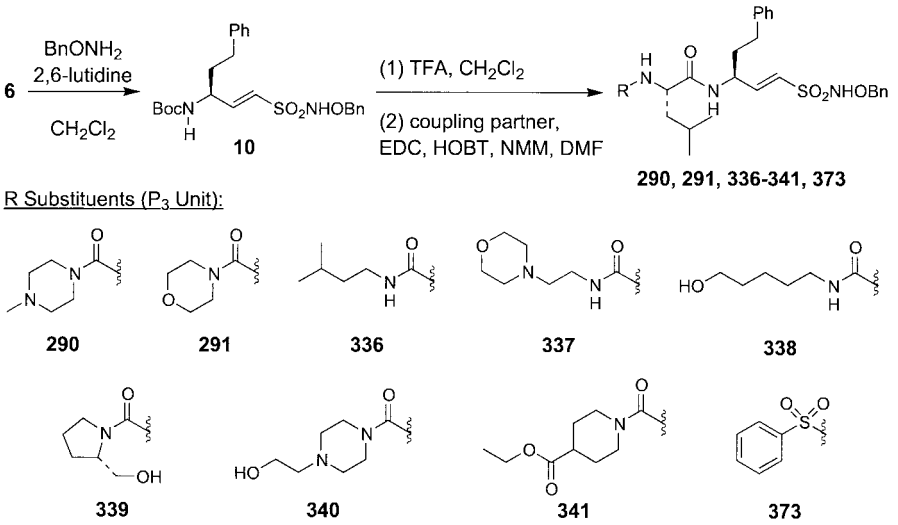

FIG. 4. Scheme for the synthesis of vinyl sulfonamides with Leu at the P_2 position and Phe at the P_1 position. For all other abbreviations, see the legend to Fig. 2.

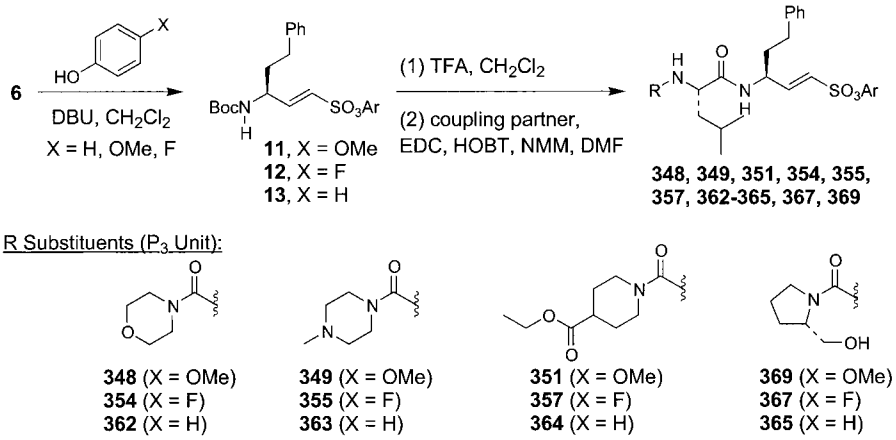

FIG. 5. Scheme for the synthesis of vinyl sulfonate esters with Leu at the P_2 position and homoPhe at the P_1 position. DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; OMe, *O*-methyl. For all other abbreviations, see the legend to Fig. 2.

studies showed that the Leu-homoPhe peptide had strong activity and that potency was imparted by alterations of amino (P_3)- and carboxy (P_1')-terminal constituents of vinyl sulfone inhibitors (Fig. 1). Consistent with prior results, compounds with the core sequence Phe-homoPhe offered modest activity against falcipain-2 (Table 1); alterations at position P_3 in these compounds had relatively little impact on activity. Compounds with the core sequence Phe-*O*-(phenyl)Ser had similar activities, with IC_{50}s for the inhibition of falcipain-2 in the mid- to high-nanomolar range (Table 1).

We next tested an additional 30 compounds, all of which contained the core sequence Leu-homoPhe and which differed at the P_3 and P_1' positions. Three different constituents at P_1' generated phenyl vinyl sulfones, vinyl sulfonamides, and vinyl sulfonate esters. The Leu-homoPhe compounds were generally very active against falcipain-2, with IC_{50}s mostly in the high-picomolar to low-nanomolar range (Table 2). Considering compounds that were identical except for the P_1' substituent, the general rank order of activity against falcipain-2 was vinyl sulfonate esters > vinyl sulfonamides > phenyl vinyl sulfones (e.g., compare compounds 365, 367, or 369 with 339 and 324, or compare compounds 364, 341, and 328).

Effects of vinyl sulfones on cultured malaria parasites. Of the 30 compounds with the core sequence Leu-homoPhe, 28 had IC_{50}s against falcipain-2 below 100 nM, and these were screened for activity against cultured malaria parasites. Ring-stage parasites were treated with different concentrations of inhibitors, and after 48 h, the numbers of new ring-stage parasites in both treated and control cultures were compared. In this assay, the phenyl vinyl sulfones were the most potent inhibitors, with most tested compounds yielding IC_{50}s for the inhibition of parasite development in the low-nanomolar range

TABLE 1. Effects of R_3-Phe-homoPhe-vinyl sulfonamide-*N*-benzyloxy and R_3-Phe-*O*-(phenyl)Ser-vinyl sulfonamide-*N*-benzyloxy sulfonamides on the activity of falcipain-2[a]

Compound	R_1	R_1'	IC_{50} (nM)
308	HomoPhe	Sulfonamide	110
309	HomoPhe	Sulfonamide	120
310	HomoPhe	Sulfonamide	71
311	HomoPhe	Sulfonamide	120
312	*O*-(phenyl)Ser	Sulfonamide	120
313	*O*-(phenyl)Ser	Sulfonamide	56
314	*O*-(phenyl)Ser	Sulfonamide	290
315	*O*-(phenyl)Ser	Sulfonamide	230
316	*O*-(phenyl)Ser	Sulfonamide	130

[a] For structures, see Fig. 3.

TABLE 2. Effects of R_3-Leu-homoPhe-vinyl sulfonyl-R′ peptidyl vinyl sulfonyl compounds on the activity of falcipain-2 and development of *P. falciparum*[a]

Compound	R_1'	FP-2 IC_{50} (nM)	Culture IC_{50} (nM)
322	Sulfone	35	200
324	Sulfone	27	1,800
325	Sulfone	8.7	4.5
326	Sulfone	9.2	15
327	Sulfone	3.5	22
328	Sulfone	6.9	3.9
344	Sulfone	9.9	21
345	Sulfone	6.7	1.6
371	Sulfone	140	ND
290	Sulfonamide	2.3	4.4
291	Sulfonamide	2.2	46
336	Sulfonamide	25	>10,000
337	Sulfonamide	14	1,500
338	Sulfonamide	16	2,000
339	Sulfonamide	3.6	220
340	Sulfonamide	2.5	22
341	Sulfonamide	2.2	1.6
373	Sulfonamide	16	3,300
348	Sulfonate ester	0.7	14
349	Sulfonate ester	1.9	35
351	Sulfonate ester	0.9	9.7
354	Sulfonate ester	0.7	84
355	Sulfonate ester	21	1,100
357	Sulfonate ester	0.9	120
362	Sulfonate ester	0.7	49
363	Sulfonate ester	350	ND
364	Sulfonate ester	0.7	42
365	Sulfonate ester	0.8	390
367	Sulfonate ester	0.9	394
369	Sulfonate ester	0.9	220

[a] For structures, see Fig. 2, 4, and 5. FP-2, falcipain-2; ND, not done.

TABLE 3. Second-order binding constants for binding of peptidyl vinyl sulfonyl compounds to falcipain-2 and falcipain-3

Compound	R_1'	k_{ass} (M^{-1} s^{-1})	
		Falcipain-2	Falcipain-3
325	Sulfone	21,600	39,600
326	Sulfone	29,700	31,300
327	Sulfone	78,600	57,100
328	Sulfone	111,000	127,000
344	Sulfone	54,600	61,700
345	Sulfone	102,000	55,700
290	Sulfonamide	74,700	147,000
291	Sulfonamide	411,000	407,000
340	Sulfonamide	120,000	179,000
341	Sulfonamide	245,000	711,000
348	Sulfonate ester	$>10^6$	$>10^6$
349	Sulfonate ester	288,000	160,000
351	Sulfonate ester	$>10^6$	$>10^6$
354	Sulfonate ester	$>10^6$	$>10^6$
364	Sulfonate ester	$>10^6$	$>10^6$

(Table 2). Inhibition of parasite development was consistently accompanied by the appearance of darkly stained, swollen food vacuoles, which are indicative of a block in hemoglobin hydrolysis, confirming that the inhibitors exerted their antimalarial effects by blocking food vacuole hemoglobinases. The other tested compounds were generally somewhat less active against cultured parasites, despite their having greater activity against falcipain-2. With compounds with otherwise identical structures, the rank order of antiparasitic activity was generally phenyl vinyl sulfones $\approx$ vinyl sulfonamides $>$ vinyl sulfonate esters, the opposite of the order seen for activity against falcipain-2. Within each compound class, there were fairly good correlations between inhibition of falcipain-2 and antiparasitic activity; these correlations were quite strong after the elimination of two compounds with unusually poor inhibitory activity against cultured parasites, presumably due to limited access to intracellular protease targets (r^2 was 0.943 for the sulfones, excluding compound 324; r^2 was 0.993 for the sulfonamides, excluding compound 373; r^2 was 0.802 for the sulfonate esters).

Second-order rate constants for inhibition of falcipain-2 and falcipain-3. Fifteen vinyl sulfones that were low-nanomolar inhibitors of both falcipain-2 activity and parasite development were further analyzed for inhibition of falcipain-2 and the related *P. falciparum* protease falcipain-3. The second-order rate constants (k_{ass}) for inhibition of falcipain-2 or falcipain-3 by the selected vinyl sulfonyl inhibitors were determined by the progress curve method under pseudo-first-order conditions (41). Inhibition rate constants for falcipain-2 and falcipain-3 were generally similar. As seen with IC_{50} determinations, the vinyl sulfonate esters were the most potent inhibitors, with k_{ass} values against both proteases being $>10^5$ for all five tested compounds (Table 3). The second-order rate constants for the phenyl vinyl sulfones and vinyl sulfonamides were lower, but they were consistently $>10^4$. P_3 substituents also had a marked impact on inhibitory kinetics, but a general trend was not apparent. In some cases (e.g., with compounds 328, 341, and 345) compounds containing P_3 groups with an extension at the 4 position yielded higher second-order rate constants than those lacking such a moiety (e.g., compounds 290 and 325). Further exploration at that position may be warranted.

DISCUSSION

Earlier studies showed that peptidyl vinyl sulfones were potent inhibitors of falcipain-2 (30) and that one compound cured *Plasmodium vinckei*-infected mice (22). To better characterize SAR for both enzyme and parasite inhibition, we synthesized and evaluated 39 new peptidyl vinyl sulfones, vinyl sulfonate esters, and vinyl sulfonamides. Consistent with prior results, we showed that inhibitors with the core sequence Leu-homoPhe provided potent inhibition of falcipain-2, falcipain-3, and cultured malaria parasites. Additional evaluations of compounds with the Leu-homoPhe core highlighted key SAR determinants at the P_3 and P_1' constituents of the inhibitors.

It is noteworthy that second-order rate constants for inhibition of falcipain-2 and falcipain-3 were consistently quite similar. Prior studies with peptide substrates have shown the enzymes to have similar specificities (with marked preference for substrates with Leu at P_2), but falcipain-3 was much less active than falcipain-2 against these substrates. It is not clear why this difference is not seen with peptidyl inhibitors. Rather, second-order rate constants for the inhibition of falcipain-3 were generally somewhat higher than those for falcipain-2, though in most cases the differences between the two proteases were fairly small. This finding is important for two reasons. First, it suggests that our prior screens of compounds against falcipain-2 were probably adequate in predicting inhibition of both principal *P. falciparum* cysteine protease hemoglobinases. Second, it argues that drug discovery against these two enzymes will likely not be complicated by the need to develop separate inhibitors of each protease.

Considering SAR for enzyme inhibition, vinyl sulfonate esters, and vinyl sulfonamides offered improved potency over phenyl vinyl sulfones. Little is known about the "prime-side" specificities of cysteine proteases, but in the case of the falcipains, it appears that larger P_1' substituents improve inhibitor binding. For example, it was previously noted that a naphthyl vinyl sulfone was more active both against falcipain-2 and against cultured *P. falciparum* parasites than was the corresponding phenyl vinyl sulfone (22). P_3 substituents also had an impact on activity against falcipain-2 and falcipain-3, although for compounds with otherwise identical structures, the second-order rate constants varied by less than an order of magnitude among compounds with different P_3 substituents. Available methodologies for evaluating substrate specificities of cysteine proteases, including peptidyl substrates bound to fluorogenic reporter molecules, are mostly limited to analyses of "non-prime" specificity. However, newer combinatorial approaches to enzyme evaluation (15), including methods for screening prime-side specificity (36), should improve our ability to characterize the specificities of proteases and thereby predict optimal inhibitor interactions.

Considering SAR for the inhibition of parasite development, it is noteworthy that the potency of inhibition of falcipain-2 and falcipain-3 is not an ideal predictor of activity against parasites. Clearly, the cysteine proteases must be inhibited for a vinyl sulfone to be active against the parasites, and much more potent inhibitors are generally better antimalarials (e.g., morpholine urea-Leu-homoPhe-phenyl vinyl sulfone was about an order of magnitude more active against both falcipain-2 and cultured parasites than was the corresponding Phe-homoPhe

compound) (30). However, among very potent inhibitors, additional determinants of SAR come into play. Considering our results, it appears that vinyl sulfonamides and vinyl sulfones tend to be more active than the vinyl sulfonate esters against the parasites. Regarding substitution of the aryl ring of vinyl sulfonate esters, there is a clear pattern of activity against the parasite cultures in rank order (-OMe > -H > -F, contrary to results of the enzymatic assays. Concerning the P_3 substituents, of the six compounds with the highest antiparasitic activities, three contained an isonipecotic ester moiety (compounds 328, 341, and 351) while the other three contained a tertiary amine (compounds 290, 325, and 345). Indeed, compounds containing the isonipecotic ester moiety were particularly effective against both the enzymes and cultured parasites. However, other compounds that exhibited high activity against the enzymes did not retain potency against the parasites. For example, those compounds containing a prolinol substituent at P_3 (compounds 324, 339, 365, 367, and 369) were typically very potent against the enzymes but showed relatively poor antiparasitic activity. The origin of these preferences is not yet clear.

Peptidyl vinyl sulfones were originally developed as inhibitors of papain (14, 19) and were later optimized as specific inhibitors of human cysteine proteases (3, 23). Related phenyl vinyl sulfones also inhibit cruzain, a cysteine protease of *Trypanosoma cruzi* (31, 32, 35), and one of these compounds is currently undergoing preclinical studies for the treatment of Chagas' disease (34). While the clinical use of peptidyl protease inhibitors is potentially problematic due to limited bioavailability or poor pharmacokinetics, there are numerous recent reports of peptidyl inhibitors of renin (17), thrombin (11), leukocyte elastase (42), neutrophil elastase (8), and human immunodeficiency virus type 1 protease (2, 16, 40) that are biologically active after oral administration. Considering these data, the reported in vivo efficacy of an orally administered vinyl sulfone against murine malaria (22), and our demonstration here of marked antimalarial potency, additional evaluation of vinyl sulfonyl derivatives as potential antimalarial cysteine protease inhibitors seems appropriate. More broadly, our data provide the most detailed assessment to date of the SAR for inhibition of the falcipains and of parasite development and thereby should aid the progress of peptide- and nonpeptide-based drug discovery efforts directed against plasmodial cysteine proteases.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (grants AI 35800 and AI 35707) and the Medicines for Malaria Venture. P.Y.C. was supported by a postdoctoral fellowship from the Susan G. Komen Breast Cancer Foundation.

REFERENCES

1. **Banerjee, R., J. Liu, W. Beatty, L. Pelosof, M. Klemba, and D. E. Goldberg.** 2002. Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. Proc. Natl. Acad. Sci. USA **99:**990–995.
2. **Bold, G., A. Fassler, H. G. Capraro, R. Cozens, T. Klimkait, J. Lazdins, J. Mestan, B. Poncioni, J. Rosel, D. Stover, M. Tintelnot-Blomley, F. Acemoglu, W. Beck, E. Boss, M. Eschbach, T. Hurlimann, E. Masso, S. Roussel, K. Ucci-Stoll, D. Wyss, and M. Lang.** 1998. New aza-dipeptide analogues as potent and orally absorbed HIV-1 protease inhibitors: candidates for clinical development. J. Med. Chem. **41:**3387–3401.
3. **Brömme, D., J. L. Klaus, K. Okamoto, D. Rasnick, and J. T. Palmer.** 1996. Peptidyl vinyl sulfones: a new class of potent and selective cysteine protease inhibitors. S_2P_2 specificity of human cathepsin O2 in comparison with cathepsins S and L. Biochem. J. **315:**85–89.
4. **Caffrey, C. R., E. Hansell, K. D. Lucas, L. S. Brinen, A. Alvarez-Hernandez, J. Cheng, S. L. Gwaltney II, W. R. Roush, Y. D. Stierhof, M. Bogyo, D. Steverding, and J. H. McKerrow.** 2001. Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. Mol. Biochem. Parasitol. **118:**61–73.
5. **Carretero, J. C., M. Demillequand, and L. Ghosez.** 1987. Synthesis of α,β-unsaturated sulphonates via the Wittig-Horner reaction. Tetrahedron **43:** 5125–5134.
6. **Chatterjee, S., M. A. Ator, D. Bozyczko-Coyne, K. Josef, G. Wells, R. Tripathy, M. Iqbal, R. Bihovsky, S. E. Senadhi, S. Mallya, T. M. O'Kane, B. A. McKenna, R. Siman, and J. P. Mallamo.** 1997. Synthesis and biological activity of a series of potent fluoromethyl ketone inhibitors of recombinant human calpain I. J. Med. Chem. **40:**3820–3828.
7. **Cherney, R. J., and L. Wang.** 1996. Efficient Mitsunobu reactions with *N*-phenylfluorenyl or *N*-trityl serine esters. J. Org. Chem. **61:**2544–2546.
8. **Edwards, P. D., D. W. Andisik, C. A. Bryant, B. Ewing, B. Gomes, J. J. Lewis, D. Rakiewicz, G. Steelman, A. Strimpler, D. A. Trainor, P. A. Tuthill, R. C. Mauger, C. A. Veale, R. A. Wildonger, J. C. Williams, D. J. Wolanin, and M. Zottola.** 1997. Discovery and biological activity of orally active peptidyl trifluoromethyl ketone inhibitors of human neutrophil elastase. J. Med. Chem. **40:**1876–1885.
9. **Eggleson, K. K., K. L. Duffin, and D. E. Goldberg.** 1999. Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. J. Biol. Chem. **274:**32411–32417.
10. **Enders, D., S. von Berg, and B. Jandeleit.** 2000. Diethyl [(phenylsulfonyl) methyl]phosphonate. Org. Synth. **78:**169–175.
11. **Feng, D.-M., S. J. Gardell, S. D. Lewis, M. G. Bock, Z. Chen, R. M. Freidinger, A. M. Naylor-Olsen, H. G. Ramjit, R. Woltmann, E. P. Baskin, J. L. Lynch, R. Lucas, J. A. Shafer, K. B. Dancheck, I.-W. Chen, S.-S. Mao, J. A. Krueger, T. R. Hare, A. M. Mulichak, and J. P. Vacca.** 1997. Discovery of a novel, selective, and orally bioavailable class of thrombin inhibitors incorporating aminopyridyl moieties at the P_1 position. J. Med. Chem. **40:**3726–3733.
12. **Francis, S. E., D. J. Sullivan, Jr., and D. E. Goldberg.** 1997. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. Annu. Rev. Microbiol. **51:**97–123.
13. **Gennari, C., B. Salom, D. Potenza, and A. Williams.** 1994. Synthesis of sulfonamido-pseudopeptides—new chiral unnatural oligomers. Angew. Chem. Int. Ed. Engl. **33:**2067–2069.
14. **Hanzlik, R. P., and S. A. Thompson.** 1984. Vinylogous amino acid esters: a new class of inactivators for thiol proteases. J. Med. Chem. **27:**711–712.
15. **Harris, J. L., B. J. Backes, F. Leonetti, S. Mahrus, J. A. Ellman, and C. S. Craik.** 2000. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc. Natl. Acad. Sci. USA **97:**7754–7759.
16. **Kempf, D. J., H. L. Sham, K. C. Marsh, C. A. Flentge, D. Betebenner, B. E. Green, E. McDonald, S. Vasavanonda, A. Saldivar, N. E. Wideburg, W. M. Kati, L. Ruiz, C. Zhao, L. Fino, J. Patterson, A. Molla, J. J. Plattner, and D. W. Norbeck.** 1998. Discovery of ritonavir, a potent inhibitor of HIV protease with high oral bioavailability and clinical efficacy. J. Med. Chem. **41:**602–617.
17. **Kleinert, H. D., S. H. Rosenberg, W. R. Baker, H. H. Stein, V. Klinghofer, J. Barlow, K. Spina, J. Polakowski, P. Kovar, J. Cohen, and J. Denissen.** 1992. Discovery of a peptide-based renin inhibitor with oral bioavailability and efficacy. Science **257:**1940–1943.
18. **Lambros, C., and J. P. Vandenberg.** 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. **65:**418–420.
19. **Liu, S., and R. P. Hanzlik.** 1992. Structure-activity relationships for inhibition of papain by peptide Michael acceptors. J. Med. Chem. **35:**1067–1075.
20. **McKerrow, J. H., E. Sun, P. J. Rosenthal, and J. Bouvier.** 1993. The proteases and pathogenicity of parasitic protozoa. Annu. Rev. Microbiol. **47:** 821–853.
21. **Musicki, B., and T. S. Widlanski.** 1991. Synthesis of nucleoside sulfonates and sulfones. Tetrahedron Lett. **32:**1267–1270.
22. **Olson, J. E., G. K. Lee, A. Semenov, and P. J. Rosenthal.** 1999. Antimalarial effects in mice of orally administered peptidyl cysteine protease inhibitors. Bioorg. Med. Chem. **7:**633–638.
23. **Palmer, J. T., D. Rasnick, J. L. Klaus, and D. Bromme.** 1995. Vinyl sulfones as mechanism-based cysteine protease inhibitors. J. Med. Chem. **38:**3193–3196.
24. **Reynolds, R. C., P. A. Crooks, J. A. Maddry, M. S. Akhtar, J. A. Montgomery, and J. A. Secrist III.** 1992. Synthesis of thymidine dimers containing internucleoside sulfonate and sulfonamide linkages. J. Org. Chem. **57:**2983–2985.
25. **Ridley, R. G.** 2002. Medical need, scientific opportunity and the drive for antimalarial drugs. Nature **415:**686–693.
26. **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. Investig. **82:**1560–1566.
27. **Rosenthal, P. J.** 2002. Hydrolysis of erythrocyte proteins by proteases of malaria parasites. Curr. Opin. Hematol. **9:**140–145.

28. **Rosenthal, P. J., and R. G. Nelson.** 1992. Isolation and characterization of a cysteine proteinase gene of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **51:**143–152.
29. **Rosenthal, P. J., G. K. Lee, and R. E. Smith.** 1993. Inhibition of a *Plasmodium vinckei* cysteine proteinase cures murine malaria. J. Clin. Investig. **91:**1052–1056.
30. **Rosenthal, P. J., J. E. Olson, G. K. Lee, J. T. Palmer, J. L. Klaus, and D. Rasnick.** 1996. Antimalarial effects of vinyl sulfone cysteine proteinase inhibitors. Antimicrob. Agents Chemother. **40:**1600–1603.
31. **Roush, W. R., J. Cheng, B. Knapp-Reed, A. Alvarez-Hernandez, J. H. McKerrow, E. Hansell, and J. C. Engel.** 2001. Potent second generation vinyl sulfonamide inhibitors of the trypanosomal cysteine protease cruzain. Bioorg. Med. Chem. Lett. **11:**2759–2762.
32. **Roush, W. R., S. L. Gwaltney II, J. Cheng, K. A. Scheidt, J. H. McKerrow, and E. Hansell.** 1998. Vinyl sulfonate esters and vinyl sulfonamides: potent, irreversible inhibitors of cysteine proteases. J. Am. Chem. Soc. **120:**10994–10995.
33. **Sachs, J., and P. Malaney.** 2002. The economic and social burden of malaria. Nature **415:**680–685.
34. **Sajid, M., and J. H. McKerrow.** 2002. Cysteine proteases of parasitic organisms. Mol. Biochem. Parasitol. **120:**1–21.
35. **Scheidt, K. A., W. R. Roush, J. H. McKerrow, P. M. Selzer, E. Hansell, and P. J. Rosenthal.** 1998. Structure-based design, synthesis and evaluation of conformationally constrained cysteine protease inhibitors. Bioorg. Med. Chem. **6:**2477–2494.
36. **Serveau, C., G. Lalmanach, M. A. Juliano, J. Scharfstein, L. Juliano, and F. Gauthier.** 1996. Investigation of the substrate specificity of cruzipain, the major cysteine proteinase of Trypanosoma cruzi, through the use of cystatin-derived substrates and inhibitors. Biochem. J. **313:**951–956.
37. **Shenai, B. R., and P. J. Rosenthal.** 2002. Reducing requirements for hemoglobin hydrolysis by *Plasmodium falciparum* cysteine proteases. Mol. Biochem. Parasitol. **122:**99–104.
38. **Shenai, B. R., P. S. Sijwali, A. Singh, and P. J. Rosenthal.** 2000. Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*. J. Biol. Chem. **275:**29000–29010.
39. **Sijwali, P. S., B. R. Shenai, J. Gut, A. Singh, and P. J. Rosenthal.** 2001. Expression and characterization of the *Plasmodium falciparum* haemoglobinase falcipain-3. Biochem. J. **360:**481–489.
40. **Smith, A. B., III, R. Hirschmann, A. Pasternak, W. Yao, P. A. Sprengeler, M. K. Holloway, L. C. Kuo, Z. Chen, P. L. Darke, and W. A. Schleif.** 1997. An orally bioavailable pyrrolinone inhibitor of HIV-1 protease: computational analysis and X-ray crystal structure of the enzyme complex. J. Med. Chem. **40:**2440–2444.
41. **Tian, W. X., and C. L. Tsou.** 1982. Determination of the rate constant of enzyme modification by measuring the substrate reaction in the presence of the modifier. Biochemistry **21:**1028–1032.
42. **Veale, C. A., P. R. Bernstein, C. M. Bohnert, F. J. Brown, C. Bryant, J. R. Damewood, Jr., R. Earley, S. W. Feeney, P. D. Edwards, B. Gomes, J. M. Hulsizer, B. J. Kosmider, R. D. Krell, G. Moore, T. W. Salcedo, A. Shaw, D. S. Silberstein, G. B. Steelman, M. Stein, A. Strimpler, R. M. Thomas, E. P. Vacek, J. C. Williams, D. J. Wolanin, and S. Woolson.** 1997. Orally active trifluoromethyl ketone inhibitors of human leukocyte elastase. J. Med. Chem. **40:**3173–3181.