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On-Bead Screening of a Combinatorial Fumaric Acid Derived Peptide Library Yields Antiplasmodial Cysteine Protease Inhibitors with Unusual Peptide Sequences

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A new class of cysteine protease inhibitors based on fumaric acid derived oligopeptides was successfully identified from a high-throughput screening of a solid-phase bound combinatorial library. As target enzymes falcipain and rhodesain were used, which play important roles in the life cycles of the parasites which cause malaria (*Plasmodium falciparum*) and African sleeping sickness (*Trypanosoma brucei rhodesiense*). The best inhibitors with unusual amino acid sequences not reported before for this type of enzyme were also fully analyzed in detail in solution. K_i values in the lower micromolar and even nanomolar region were found. Some inhibitors are even active against plasmodia and show good selectivity relative to other enzymes. Also the mechanism of action was studied and could be shown to be irreversible inhibition.

Introduction

The burden of tropical diseases of humans caused by protozoan parasites is large, both in terms of mortality and morbidity and because these diseases impede economic growth and prosperity.[1] Diseases such as malaria (caused by various species of *Plasmodium*) and African trypanosomiasis (sleeping sickness caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*) are among the most severe.[2,3] Late-stage trypanosomiasis is characterized by somnolence and coma, leading invariably to death, if untreated. Chemotherapy depends principally on drugs developed decades ago that lack adequate efficacy and cause serious side effects. Further, the emergence of drug-resistant *Trypanosoma* strains has been reported.[2] Similarly in malaria,[4] increasing resistance[5] of malaria parasites to antimalarial drugs, the lack of highly effective vaccines, and inadequate control of mosquito vectors demand new approaches to drug development.

In both diseases one promising strategy to develop new drugs has been to target parasite cysteine proteases.[3,6] These enzymes, termed rhodesain[7] (RD) in *T. b. rhodesiense*, brucipain in *T. brucei brucei* (infective to animals), and falcipains[8] (falcipain-1, falcipain-2, falcipain-2′, and falcipain-3)[9,10] in *Plasmodium falciparum*, belong to the cathepsin L subfamily of the papain family (clan CA, family C1; CAC1) of cysteine proteases.[11] Cysteine protease inhibitors have been shown to kill African trypanosomes *in vitro* and in animal models of the disease.[3] Proteases of malaria parasites play pivotal roles in the processes of host erythrocyte rupture, erythrocyte invasion, and hemoglobin degradation. Treatment with cysteine protease inhibitors blocks hemoglobin hydrolysis and development of the parasite.[12] While falcipain-1 is suggested to play a principal role in nonerythrocytic stages,[13] falcipain-2 and falcipain-3 are likely the major hemoglobinases in the food vacuole of erythrocytic parasites.[14] Therefore, the inhibition of cysteine proteases presents a promising strategy for combating these infections. However, no drugs that target these proteases are yet on the market.

Proteases specifically recognize their protein or oligopeptide substrate, and knowledge about substrate specificity aids the development of inhibitors. This information can be obtained by using combinatorial libraries of labeled peptides.[15,16] On the other hand, combinatorial libraries of small molecules prepared with mechanism-based scaffolds have resulted in the identification of potent inhibitors of various proteases.[17,18] Such inhibitors, if cell-permeable, can serve as lead compounds for the development of new drugs or as biochemical tools to study the function of the respective proteases *in vitro* and *in vivo*.

In this paper we describe the synthesis of a combinatorial heptapeptide library containing fumaric acid as an electrophilic building block which can covalently block the active site Cys residue of falcipain-2 and rhodesain via a Michael-type reaction. Compounds from this library also showed promising antiplasmodial activity *in vitro*.

Several efficient irreversible inhibitors of proteases based on activated olefins are known. For example, the fumaric acid derivative of the well-known epoxysuccinyl peptide (*S*,*S*)-epoxysuccinyl-(*S*)-leucylamido(4-guanidino)butane (E-64), the papain inhibitor DC-11,[19] has been shown to irreversibly block CAC1 cysteine proteases, and recently related peptidyl ene diones[20] and azapeptide Michael acceptors[21] have been identified as potent inactivators of cysteine proteases. Other

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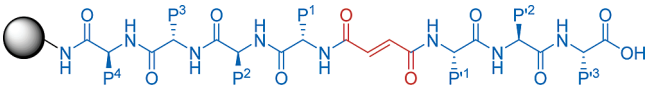
Table 1. Composition of the Heptapeptide Fumaric Acid Diamide Library[a]



P4	P3	P2	P1	P1′	P2′	P3′
Val	Val	Leu	Gln	Gly	Gly	Phe
	Ala	Ile	Asn	Ala		
	Leu	Phe	C1-NHAc			
		Chg	Ala			
		Phg	Phe			

[a] Due to the insertion of the fumaric acid moiety the definition of the amino acid residues as P1, P2, P1′, P2′, etc. does not necessarily implicate binding of these residues into the respective substrate binding pockets (S1, S2, S1′, S2′, etc.) of the enzyme.

fumaric acid derivatives have also been tested against these proteases.[22,23] In order to further explore the suitability of fumaric acid derivatives, we established a synthetic strategy for a fumaric acid diamide library and developed on-bead screening assays for falcipain-2 and rhodesain. This allowed fast high-throughput screening and selective identification of new inhibitors with amino acids unusual for cathepsin L-like cysteine proteases. Several inhibitors identified by on-bead screening of the library were synthesized in larger scale, and inhibition constants against the target enzymes as well as *in vitro* antiparasitic activities were determined.

Results

Library Design and Synthesis. The library was designed according to Table 1. The selection of amino acids mainly accounted for the known preference of CAC1 proteases for hydrophobic amino acids in the P2 position (Leu, Ile, Phe, Chg, Phg). The unnatural amino acids cyclohexylglycine (Chg) and phenylglycine (Phg) were chosen in order to enhance proteolytic stability. For P3 different nonpolar amino acids were used (Val, Ala, Leu) whereas P4 as well as the primed-site amino acids were held constant or only marginally varied in P1′ (Gly, Ala). The designation of residues as P1, P1′, etc. is simply used here as a convenient numbering scheme but does not imply binding of these residues into the respective substrate binding pockets (S1, S2, S1′, S2′, etc.) of the enzymes. Due to the central fumarate core the residues are shifted with respect to a normal peptidic substrate and might very well occupy different binding pockets.

CAC1 cysteine proteases strongly prefer Arg in the P1 position. A library which includes Arg as amino acid would most likely lead to a hit list dominated by this amino acid. In order to avoid this and to find new amino acids possibly fitting into the S1 binding site, we did not use Arg, but instead either the aromatic amino acid Phe, Ala, or amino acids with amide side chains (Gln, Asn, C1-NHAc) were chosen. This library design with amino acids such as Gln or Asn in the P1-position offers the advantage that the inhibitors can also be tested against viral cysteine proteases.[24] However, as published recently, falcipains accommodate Gln in the P1-position as well.[15] In total, the library consisted of 150 compounds (a complete list of all library members can be found in the Supporting Information).

Synthesis of the Library. The solid-phase synthesis of the inhibitor library (Scheme 1) was performed by using the split and mix method[25] on amino functionalized PEGA resin.[a] The use of PEGA resin is essential as initial experiments showed. The screening is performed in aqueous solutions so that the applied resin must have good swelling properties in polar solvents. On-bead enzyme assays described in the literature (*vide infra*) used libraries based on TentaGel[26] or PEGA resin.[27,28] Both resins contain polyethylene glycol (PEG) chains and therefore swell efficiently in water so that large biomolecules like enzymes can reach the resin-bound compounds. However, TentaGel, a polystyrene resin that is grafted with PEG chains, shows a weak but nevertheless existing interaction with proteins which can interfere with the screening results.[29] Thus, we decided to use the amino PEGA resin that is completely based on PEG chains and therefore is virtually inert toward proteins. We used the IRORI technique with radio frequency tagged micro chips to be able to determine the amino acid sequence during and after the synthesis.[30] Each of the 150 members was synthesized on 30 mg of resin in a MikroKan that was scanned and redistributed to individual reactors after each coupling step to obtain the combinatorial library spatially separated. The coupling steps were monitored by the Kaiser test[31] for free amino functions and the malachite green test[32] for carboxylates. For all coupling reactions HCTU was used as coupling agent, NMM as base, and DMF as solvent. Every coupling step was performed twice overnight. The first four amino acids were introduced by the standard Fmoc strategy, and the protecting group was removed with a solution of 20% piperidine in DMF. Then the monoprotected fumaric acid **2** (Scheme 2) was introduced and deprotected with 50% TFA in dichloromethane. The other peptide arm was synthesized via an N- to C-coupling strategy using *tert*-butyl ester protected amino acids with free amino functions.[33] The ester group was cleaved with 50% TFA in dichloromethane after each coupling step. Finally, the whole library was washed thoroughly with DMF, methanol, and dichloromethane.

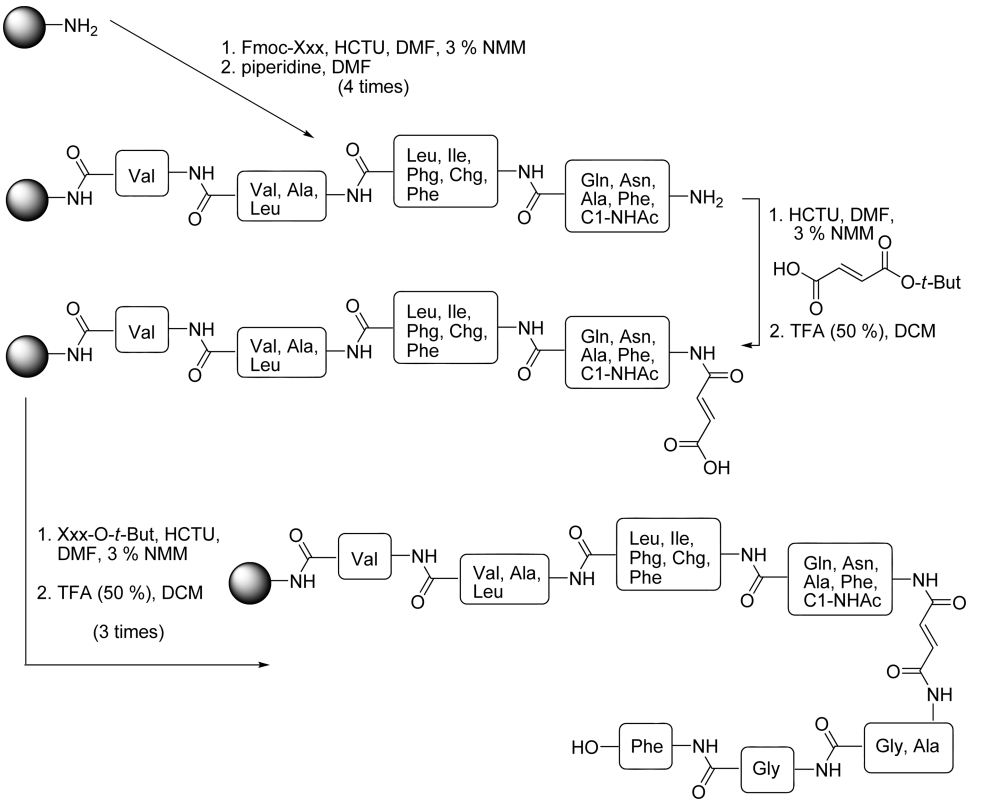
The monoprotected fumaric acid building block **2** was synthesized as shown in Scheme 2. Fumaric acid was converted to the monochloride with 1 equiv of oxalyl chloride in dichloromethane. This acid chloride was reacted with potassium *tert*-butylate to obtain the *tert*-butyl ester **2**.

All of the amino acids used for the synthesis of the library were commercially available except the unnatural amino acid Fmoc-C1-NHAc **7**. Its synthesis is shown in Scheme 3 starting from Cbz-protected Asn **3**. The first step is a literature known Hofmann degradation.[34] Then the free amine was acetylated with 1 equiv of acetic anhydride. In the last steps the amino function was deprotected with H_2 and Pd/C, and the Fmoc group was introduced.

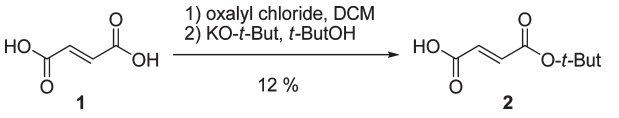
On-Bead Enzyme Assays. There are several on-bead enzyme assays described in the literature for different enzymes. Assays are described for the trypanothione reductase,[35] the aldose reductase,[36] the human serine racemase,[37] the protein disulfide isomerase,[38] and Brk protein tyrosine kinase,[39] but the best investigated enzymes are proteases. On-bead assays have been described for all four classes of proteases: metallo, aspartic, serine, and cysteine proteases.[40] In these assays in

[a] Abbreviations: AMC, aminomethylcoumarin; HCTU, 2-(6-chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate; IRORI, company which developed the radiofrequency tagging technology used here; NMM, *N*-methylmorpholine; MBHA resin, 4-methylbenzhydrylamine hydrochloride salt resin; PEGA resin, beaded polyethylene glycol polyacrylamide copolymer; PEG, polyethylene glycol; TFMSA, trifluoromethanesulfonic acid.

Scheme 1. Synthesis of the Fumaric Acid Heptapetide Library



Scheme 2. Synthesis of Fumaric Acid *tert*-Butyl Ester **2**



general a combinatorial library is incubated with an enzyme of interest, and the hits are detected by colorimetric or fluorogenic tools, then sorted, and finally analyzed by Edman sequencing, MALDI-TOF mass spectrometry, or MAS NMR spectroscopy. There are both protease substrate specificity assays and inhibitor screening assays based on fluorescence resonance energy transfer (FRET). Substrate specificity assays are based on libraries of FRET−substrates in the form of one-bead−one-compound libraries. These assays can provide information about the preferences for the hydrolysis of peptide sequences by a given enzyme. During incubation of the library with a protease, some beads show fluorescence due to a fast cleavage of the attached substrates by the enzyme.

In contrast, inhibitor screening assays so far have been based on one-bead−two-compound libraries: Each bead has a good FRET−substrate and one member of the library of potential inhibitors attached. After incubation with a protease, nonfluorescent beads indicate inhibitors.[41] This methodology requires the synthesis of both the inhibitor and the FRET−substrate on the bead, which increases the synthetic effort and also limits the library screening to one type of substrate/enzyme combination. Furthermore, all on-bead assays reported so far, whether substrate specificity assays or inhibitor screenings, provide only qualitative yes/no information. One can only decide whether there is a hit or not due to the occurrence of fluorescence or color of individual beads.

We chose a different approach here. Our screening assay is based on a one-bead−one-compound library of potential inhibitors. As the IRORI-radiofrequency tagging methodology provides the individual library members spatially separated, they can be separately incubated with both substrate and enzyme in solution. Furthermore, using a microtiter plate reader the assay can provide quantitative inhibition data for each library member. Individual library members can then be compared, and an initial structure−activity relationship can be derived. After incubation of the library with the target enzymes (falcipain-2 or rhodesain) for 12 h at 9 °C the substrate (Cbz-Phe-Arg-AMC, 40 μM) was added, and the residual enzyme activity was monitored by fluorescence spectroscopy. The enzyme hydrolyzes the substrate, thus liberating the fluorescent aminomethylcoumarin (AMC). The more efficient the inhibition, the lower the fluorescence increase. With this method we obtained quantitative on-bead inhibition data for all 150 inhibitors in the library for both falcipain-2 and rhodesain. The inhibition for both proteases ranged between 7% and 77% under these experimental conditions. Hence, our library spanned the whole range from poor to good inhibitors. Since the K_m values of the substrate used (Cbz-Phe-Arg-AMC) are quite different for the target enzymes (falcipain-2, 21.5 μM; rhodesain, 827 nM; respectively), the percentages of inhibition for the two enzymes found in the on-bead screenings cannot be compared directly. The substrate is a much better competitor in the case of rhodesain due to its higher affinity for this enzyme, so that, e.g., 25% inhibition of rhodesain means much better inhibition than 25% inhibition of falcipain-2. However, for both enzymes the results were similar concerning the preferences for the various amino acids as expected based on the homology of both proteins. The active sites are largely

Scheme 3. Synthesis of the Fmoc-Protected Amino Acid Fmoc-C1-NHAc

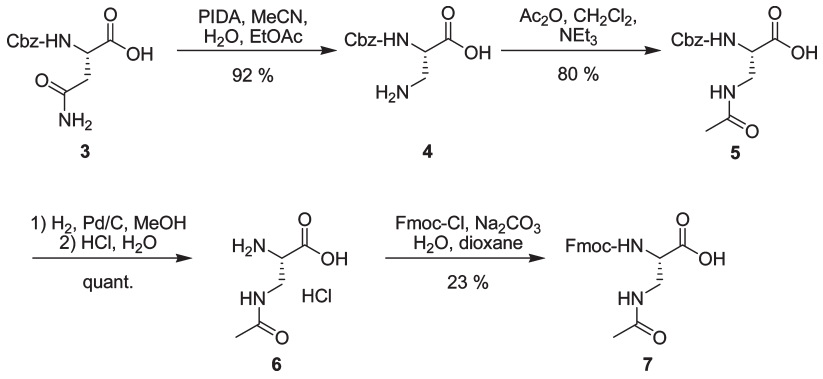


Table 2. Classification of the Inhibitors in Groups A−E by Their On-Bead Inhibitory Potential and Number of Library Members Included in Each Subset

	A	B	C	D	E
inhibition in on-bead	$I \geq 60\%$	$60\% > I \geq 50\%$	$50\% > I \geq 40\%$	$40\% > I \geq 30\%$	$30\% > I$
falcipain-2	19	45	42	29	15
rhodesain	37	34	32	24	23

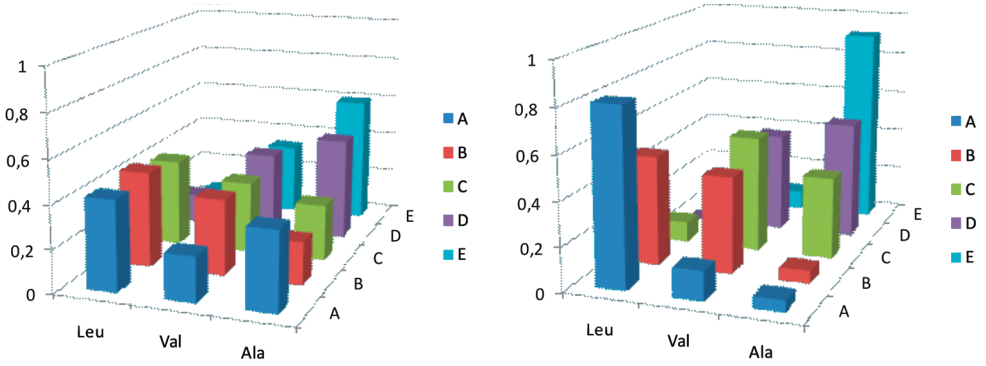


Figure 1. Relative frequencies of amino acids at P3 for falcipain-2 (left) and rhodesain (right). Subsets were defined as follows according to the inhibition I observed in the on-bead screening: subset A, $I \geq 60\%$; subset B, $60\% > I \geq 50\%$; subset C, $50\% > I \geq 40\%$; subset D, $40\% > I \geq 30\%$; subset E, $30\% > I$.

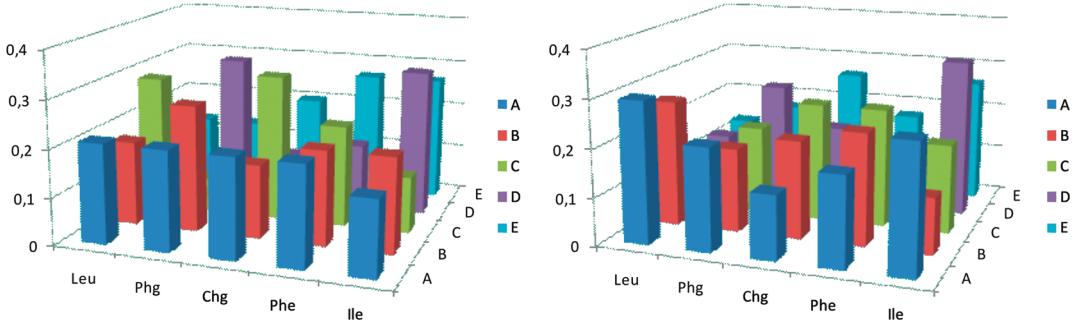


Figure 2. Relative frequencies of amino acids at P2 for falcipain-2 (left) and rhodesain (right). Subsets were defined as follows according to the inhibition I observed in the on-bead screening: subset A, $I \geq 60\%$; subset B, $60\% > I \geq 50\%$; subset C, $50\% > I \geq 40\%$; subset D, $40\% > I \geq 30\%$; subset E, $30\% > I$.

identical.[42] However, there are some differences which might be interesting as starting points to develop more specific inhibitors for just one of the two enzymes (*vide infra*).

By comparison of the quantitative inhibition data, we were furthermore able to derive some structure−activity relationships. For this, we subdivided the library into five groups, A−E, according to the inhibitory potential of the compounds (Table 2). Then the relative frequencies of the various amino acids in each of the four variable positions for each subset were analyzed.

The relative frequencies of each amino acid in position P1 to P3 are illustrated in Figures 1−3. At P3 for both enzymes Leu is the most efficient amino acid. However, this preference is much more pronounced for rhodesain than for falcipain-2. The former does not tolerate any other amino acid in P3. The inhibition significantly drops for both valine and alanine. About 80% of the most potent inhibitors (subset A) have Leu in P3 and only about 15% Val and even less for Ala. In contrast, Ala in P3 leads to mostly inactive inhibitors for rhodesain (> 95% occurrence in subset E). For

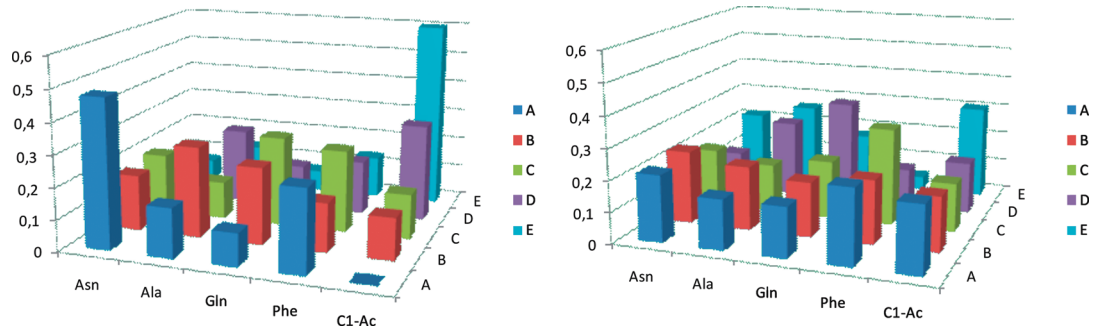


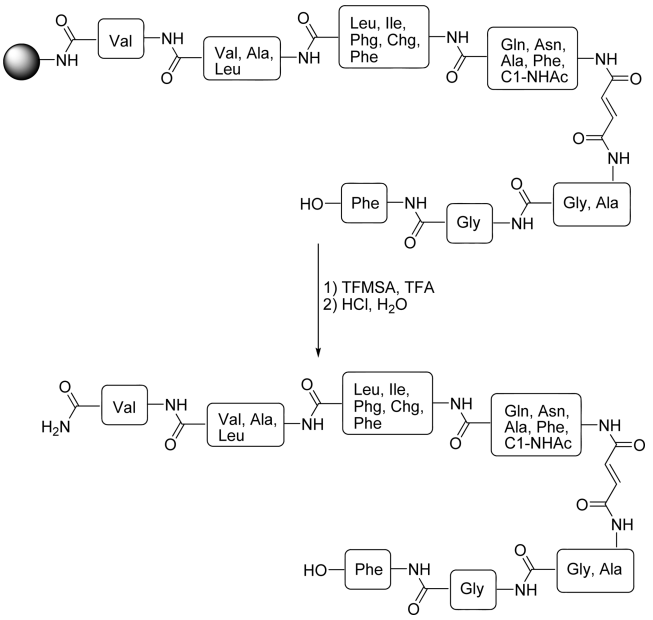
Figure 3. Relative frequencies of amino acids at P1 for falcipain-2 (left) and rhodesain (right). Subsets were defined as follows according to the inhibition *I* observed in the on-bead screening: subset A, $I \geq 60\%$; subset B, $60\% > I \geq 50\%$; subset C, $50\% > I \geq 40\%$; subset D, $40\% > I \geq 30\%$; subset E, $30\% > I$.

falciapin-2 the choice of amino acid in P3 is much less decisive. The three amino acids are more equally distributed over all five subsets. For example, in the most potent inhibitors (subset A), Ala is nearly as often found as Leu (both ca. 40%) and even Val is found in 20% of the potent inhibitors. In the moderate inhibitors (subset C) again all three amino acids are found with similar frequency. The only significant selectivity is that Leu is not found in the worst inhibitors (< 5% occurrence). Therefore, the amino acid in P3 might provide a starting point for the development of inhibitors which can efficiently differentiate between falcipain-2 and rhodesain despite their overall similarity.

In contrast to this, at P2 we did not observe any clear preferences for any amino acid in both enzymes (Figure 2). This means that all five amino acids which were included in the library at this position (Leu, Phg, Chg, Phe, Ile) are equally well accepted by both enzymes. This position is important for neither the potency of the inhibitors nor the enzyme selectivity at least with this subset of amino acids used here. This seems at the first glance to contradict results from other substrate selectivity screenings which indicate a strong preference for Leu at this position.[43] However, the two unnatural amino acids Phg and Chg we used here have not been tested in those earlier reports which concentrated on standard proteinogenic amino acids. Also, it is known from other enzymes (e.g., SARS-coronavirus main protease) that Leu can be substituted by Phg and Chg while maintaining activity.[44] Furthermore, lacking any structural data on these new inhibitors, we do not know their exact positioning in the active site of the enzymes. The fumaric acid moiety in the central part of our inhibitors might affect the binding mode of the peptide in the enzyme, causing a slightly different selectivity pattern for the individual binding sites as compared to a natural peptide substrate.

The situation for P1 is similar to that of P3 but with a reversed behavior of both enzymes (Figure 3). Rhodesain does not show any clear preference for any of the five amino acids used at P1. In contrast to this, falcipain-2 now significantly prefers Asn in P1 whereas the "reversed amide" in C1-NHAc leads to a nearly complete loss of inhibition. About 47% of the most potent inhibitors (subset A) contain Asn in P1, followed by Phe (ca. 26%) but no C1-NHAc. In contrast, nearly 60% of the worst inhibitors had C1-NHAc at P1. This is remarkable in two aspects. The chemical difference between Asn and C1-NHAc is rather small, as both contain an amide group but in a reversed order. Second, this preference for Asn and the complete intolerance for C1-NHAc by falcipain-2 but not rhodesain again might be useful as a starting point for the development of enzyme-selective inhibitors. At P1′ for both enzymes Gly is slightly preferred compared to Ala; however, the difference is not significant (data not shown).

Scheme 4. Solid-Phase Synthesis of the 14 Selected Library Members



To conclude, our combinatorial quantitative on-bead screening not only revealed new and potent inhibitors for two medically important cysteine proteases, falcipain-2 and rhodesain, but also provided insight into some interesting structure−activity relationships which might prove useful for the further development of a next generation of enzyme-selective inhibitors.

Synthesis of Selected Library-Derived Inhibitors. However, in order to validate these on-bead screening results, 14 inhibitors from the library were selected for further evaluation in solution. The selected inhibitors fulfilled the following requirements: (1) They were selected from all five subsets A−E, so that both highly potent and inactive inhibitors could be tested. (2) The best amino acids for each of the four variable positions were included. The synthesis of these inhibitors (Scheme 4) followed the synthesis of the library as described above, but this time an amino-functionalized MBHA resin was used. After the coupling of the last amino acid the peptidic compounds were cleaved from the resin under acidic conditions with 10% TFMSA in TFA and isolated by precipitation from diluted hydrochloric acid.

Table 3. Inhibition of Falcipain-2 (FP-2) and Antiplasmodial Activities of 14 Selected Library Members

no.	structure[a]	on-bead inhibition (%) of FP-2	inhibition (%) of FP-2 at 100 μM	K_i (μM), FP-2	IC_{50} (μM), *P. falciparum*
8	H_2N-Val←Leu←Leu←Asn←Fum-Gly-Gly-Phe-OH	70	92	10.1	74 ± 9^b
9	H_2N-Val←Ala←Leu←Asn←Fum-Gly-Gly-Phe-OH	41	33	67.5	nd
10	H_2N-Val←Leu←Phe←Asn←Fum-Gly-Gly-Phe-OH	70	49	nd	nd
11	H_2N-Val←Leu←Phg←Asn←Fum-Gly-Gly-Phe-OH	51	76	21.3	ni[b]
12	H_2N-Val←Leu←Leu←Phe←Fum-Gly-Gly-Phe-OH	69	100	0.8	ni[b]
13	H_2N-Val←Val←Leu←Gln←Fum-Ala-Gly-Phe-OH	48	45	nd	nd
14	H_2N-Val←Ala←Chg←Asn←Fum-Gly-Gly-Phe-OH	73	79	12.2	75 ± 9^b
15	H_2N-Val←Val←Chg←Asn←Fum-Gly-Gly-Phe-OH	61	90	5.2	32 ± 7^b
16	H_2N-Val←Val←Chg←Gln←Fum-Gly-Gly-Phe-OH	51	83	1.5	27 ± 10^b
17	H_2N-Val←Ala←Chg←C1-NHAc←Fum-Gly-Gly-Phe-OH	23	83	7.0	22 ± 4^b
18	H_2N-Val←Leu←Leu←Ala←Fum-Gly-Gly-Phe-OH	55	73	nd	ni[c]
19	H_2N-Val←Leu←Leu←Ala←Fum-Ala-Gly-Phe-OH	58	86	9.5[d]	ni[c]
20	H_2N-Val←Leu←Leu←C1-NHAc←Fum-Gly-Gly-Phe-OH	54	79	11.8[d]	ni[c]
21	H_2N-Val←Ala←Leu←C1-NHAc←Fum-Gly-Gly-Phe-OH	32	18	nd	ni[c]
	Reference Compounds				
E-64		nd	nd	0.29[e]	5.3[b,e]
CQ		nd	nd	nd	0.24[c,e]

[a] Fum, fumaric acid (O=C—HC=CH—C=O); Chg, cyclohexylglycine; Phg, phenylglycine; C1-NHAc, acetylaminoalanine; ←, peptide sequence reversed from the C- to N-terminus; nd, not determined. All values are mean values of at least two independent assays; mean standard deviations are less than 5−10% unless otherwise indicated. [b] Strain FCBR. [c] Strain W2. [d] Cbz-Leu-Arg-AMC ([S] = 25 μM, K_m = 8.4 μM) used as substrate. [e] Taken from ref 45; E-64, (S,S)-epoxy-succinyl-(S)-leucylamido(4-guanidino)butane; CQ, chloroquine.

The compounds are initially isolated as the free carboxylic acids, some of which have a limited solubility in aqueous solutions. To obtain better soluble compounds, the triethylammonium salts of the inhibitors were therefore prepared.

Biological Activities. These 14 inhibitors were tested against falcipain-2 (FP-2) and rhodesain (RD) in standard fluorescence assays as published previously.[45,46] The hydrolyses of Cbz-Phe-Arg-AMC or Cbz-Leu-Arg-AMC in the absence or presence of the respective inhibitor were measured by following the fluorescence increase due to release of AMC. As a positive control the well-known cysteine protease inhibitor E-64[47,48] and as negative control the solvent DMSO were used. In addition, inhibition was tested against mammalian analogues of these two cysteine proteases, namely, cathepsins L and B. Since falcipain-2 and rhodesain belong to the cathepsin L-like cysteine proteases, this study should also clarify the subtype selectivity of the inhibitors. With the most potent inhibitors dialysis assays were performed in order to verify the irreversible inhibition mechanism. Unfortunately, the limited long-time stability of falcipain-2 and rhodesain prevented any dialysis studies with these two enzymes. We therefore tested inhibitor **15**, which revealed to be also quite active against cathepsin B, in a dialysis experiment. These studies proved the expected irreversible inhibition mechanism, since after 3 h of continuous dialysis against assay buffer the enzyme activity did not recover (inhibitor concentration 200 μM, 74% and 73% inhibition before and after dialysis, respectively).

First, initial screenings with 100 μM inhibitor concentration were performed. For inhibitors which displayed considerable activity in this screening, dissociation constants K_i were determined in order to specify the affinities to the target enzymes.[49] Inhibitors displaying K_i values in the lower micromolar range were further tested for antiplasmodial and antitrypanosomal activities. Antiplasmodial activities were determined with the *P. falciparum* strain FCBR using a recently published assay[50] or by flow cytometry according to a previously published method using *P. falciparum* strain W2.[10] Chloroquine[51] and E-64[52] were used as positive controls, and the solvent DMSO was used as a negative control. Antitrypanosomal activities were determined against *T. b. brucei* as published earlier.[46] The well-known drugs melarsoprol, eflornithine, suramine, nifurtimox, and pentamidine were used as positive controls. All inhibition data are summarized in Tables 3, 4, and 5.

Summary of the Results. Comparing the results obtained in the on-bead screening with the standard solution assays at 100 μM inhibitor concentration, a good correlation is observed. In general, for both enzymes, rhodesain and falcipain-2, >50% inhibition in the on-bead screening results in >50% inhibition at 100 μM in solution. Even more importantly, the relative trends in the inhibition data are similar for both solid-phase-bound inhibitors and free inhibitors in solution. Therefore, we successfully could identify new and potent inhibitors using our combinatorial on-bead screening assay.

The most potent inhibitors are peptides **12**, **15**, and **16** with K_i values in the lower micromolar or even nanomolar region (K_i(**12**) = 0.8/0.2 μM, K_i(**15**) = 5.2/0.08 μM, and K_i(**16**) = 1.5/0.08, for falcipain-2/rhodesain, respectively). Again, small changes in the structure can lead to significant differences in inhibition potency as was also observed in the on-bead assay. For example, replacing Phe in P1 position in **12** against an amino acid with an amide side chain (Asn (**8**) or C1-NHAc (**20**)) leads to an at least 10-fold decrease in inhibition potency for both falcipain-2 (K_i = 10.1 and 11.8 μM, respectively) and rhodesain (K_i = 2.7 and 1.8 μM, respectively). In agreement with the results from the on-bead screening no significant differences can be found for Gly or Ala in P1′ (compare inhibitors **18** and **19**) or the exchange of an amino acid in P2 (compare inhibitors **10** and **11** with either Phe or Phg in P2 or inhibitors **9** and **14** with either Ala or Chg in P2).

Inhibitor **12** was also explicitly tested with or without added detergent to exclude that nonspecific aggregate formation[53] is the reason for the observed inhibition results (which would have been very unlikely, in light of the positive on-bead screening assay in which aggregation cannot occur,

Table 4. Inhibition of Rhodesain (RD) and Cathepsin B (CB) and Antitrypanosomal Activities of 14 Selected Library Members

no.	structure[a]	on-bead inhibition (%) of RD	inhibition (%) of RD at 100 μM	K_i (μM), RD	IC_{50} (μM), *T. b. b.*	inhibition (%) of CB at 100 μM	K_i (μM), CB
8	H_2N-Val←Leu←Leu←Asn←Fum-Gly-Gly-Phe-OH	71	63	2.7	nd	21	nd
9	H_2N-Val←Ala←Leu←Asn←Fum-Gly-Gly-Phe-OH	24	8	nd	nd	17	nd
10	H_2N-Val←Leu←Phe←Asn←Fum-Gly-Gly-Phe-OH	61	6	nd	nd	24	nd
11	H_2N-Val←Leu←Phg←Asn←Fum-Gly-Gly-Phe-OH	50	26	nd	nd	13	nd
12	H_2N-Val←Leu←Leu←Phe←Fum-Gly-Gly-Phe-OH	69	68	0.2	nd	73	22.9
13	H_2N-Val←Val←Leu←Gln←Fum-Ala-Gly-Phe-OH	64	37	nd	nd	23	nd
14	H_2N-Val←Ala←Chg←Asn←Fum-Gly-Gly-Phe-OH	26	98	0.4	nd	78	22.2
15	H_2N-Val←Val←Chg←Asn←Fum-Gly-Gly-Phe-OH	46	96	0.08	ni	100	1.4
16	H_2N-Val←Val←Chg←Gln←Fum-Gly-Gly-Phe-OH	53	99	0.08	ni	81	nd
17	H_2N-Val←Ala←Chg←C1-NHAc←Fum-Gly-Gly-Phe-OH	25	95	0.3	ni	83	12.4
18	H_2N-Val←Leu←Leu←Ala←Fum-Gly-Gly-Phe-OH	65	99	1.0	nd	38	nd
19	H_2N-Val←Leu←Leu←Ala←Fum-Ala-Gly-Phe-OH	67	96	1.3	nd	45	nd
20	H_2N-Val←Leu←Leu←C1-NHAc←Fum-Gly-Gly-Phe-OH	60	87	1.8	nd	ni	nd
21	H_2N-Val←Ala←Leu←C1-NHAc←Fum-Gly-Gly-Phe-OH	32	30	nd	nd	ni	nd
	Reference Compounds						
MS		nd	94	nd	0.0026[b]	nd	nd
EF		nd	31	nd	30.7[b]	nd	nd
SA		nd	32	nd	0.31[b]	nd	nd
NX		nd	nd	nd	3.4[b]	nd	nd
PN		nd/nd	nd/28[c]	nd	0.0029[b]	nd	nd

[a] Fum, fumaric acid (O=C—HC=CH—C=O); Chg, cyclohexylglycine; Phg, phenylglycine; C1-NHAc, acetylaminoalanine; ←, peptide sequence reversed from the C- to N-terminus; nd, not determined. All values are mean values of at least two independent assays; mean standard deviations are less than 5−10% unless otherwise indicated. [b] Taken from ref 46 [c] [I] = 200 μM. MS, melarsoprol; EF, eflornithine; SA, suramine; NX, nifurtimox; PN, pentamidine.

Table 5. Inhibition of Cathepsin B (CB) and Cathepsin L (CL) of Selected Library Members

no.	structure[a]	K_i (μM), CB	K_i (μM), CL
12	H_2N-Val←Leu←Leu←Phe←Fum-Gly-Gly-Phe-OH	22.9	34.8
14	H_2N-Val←Ala←Chg←Asn←Fum-Gly-Gly-Phe-OH	22.2	nd
15	H_2N-Val←Val←Chg←Asn←Fum-Gly-Gly-Phe-OH	1.4	37.5
17	H_2N-Val←Ala←Chg←C1-NHAc←Fum-Gly-Gly-Phe-OH	12.4	nd
18	H_2N-Val←Leu←Leu←Ala←Fum-Gly-Gly-Phe-OH	nd	30.7
19	H_2N-Val←Leu←Leu←Ala←Fum-Ala-Gly-Phe-OH	nd	33.6
20	H_2N-Val←Leu←Leu←C1-NHAc←Fum-Gly-Gly-Phe-OH	nd	40.0
21	H_2N-Val←Ala←Leu←C1-NHAc←Fum-Gly-Gly-Phe-OH	nd	20.8

[a] Fum, fumaric acid (O=C—HC=CH—C=O); Chg, cyclohexylglycine; Phg, phenylglycine; C1-NHAc, acetylaminoalanine; ←, peptide sequence reversed from the C- to N-terminus; nd, not determined. All values are mean values of at least two independent assays; mean standard deviations are less than 5−10% unless otherwise indicated.

but nevertheless we wanted to address this question explicitly). The K_i values were identical, proving that any nonspecific enzyme inhibition in solution did not occur.

With the exception of **12** which is not active against *Plasmodium*, the other most potent falcipain-2 inhibitors such as **15**−**17** also display moderate antiplasmodial activity (IC_{50} ca. 20−30 μM). In contrast, no antitrypanosomal activity can be observed, even with the most potent rhodesain inhibitors **15**, **16**, and **17**. Most likely, activity was limited either by the bioavailability of these overall rather nonpolar peptides or by problems with long-time hydrolytic stability within the assay conditions.

Some of the most potent inhibitors, namely, **12** and **15**, also show good selectivity when measured against cathepsin B (CB) or cathepsin L (CL). With the exception of inhibitor **15** which displays good inhibition of cathepsin B (K_i = 1.4 μM) all other inhibitors exhibit K_i values in the upper micromolar range (12−40 μM) against the mammalian enzymes. Especially the selectivity indices for rhodesain are quite high (CB, 18, CL, 470 (**15**); CB, 115, CL, 174 (**12**); CB, 56 (**14**); CB, 41 (**17**); CL, 31 (**18**); respectively). For falcipain the selectivity was somewhat lower; e.g., inhibitors such as **14** or **17** show intermediate activity against falcipain-2 and cathepsins.

Summary

A solid-phase-bound combinatorial library of 150 fumaric acid derived peptides was synthesized and tested as cysteine protease inhibitors against falcipain and rhodesain, essential proteases of the two parasites *P. falciparum* and *T. b. rhodesiense* responsible for severe tropical infections (malaria and sleeping sickness, respectively). The inhibitors were synthesized in macroscopic amounts using a combinatorial split-mix approach and the IRORI-radio frequency tagging technology. The inhibitors were then screened directly on-bead in a newly developed quantitative high through-put fluorescence assay with the two target enzymes. Efficient inhibition of both enzymes was observed. Based on the quantitative inhibition data for all library members thus obtained, a first structure−activity relationship for this new class of protease inhibitors was derived. Unusual combinations of amino acids, not reported before for these enzymes, were found to be most active. Selected members of the library were then

resynthesized in larger quantities and studied in detail to obtain the K_i values. The most potent inhibitors (peptides **12**, **15**, and **16**) have K_i values in the lower micromolar or even nanomolar region (K_i(**12**) = 0.8/0.2 μM, K_i(**15**) = 5.2/0.08 μM, and K_i(**16**) = 1.5/0.08 for falcipain-2/rhodesain, respectively) and are also selective relative to other enzymes such as cathepsin B or cathepsin L. The best inhibitors are also active against *Plasmodium* whereas no activity against *Trypanosoma* was found. Further studies revealed that the mechanism of action is irreversible inhibition of the enzymes.

Experimental Section

General Remarks. Reaction solvents were dried and distilled before use. All other reagents were used as obtained from Aldrich, Fluka, IRIS, or GL Biochem. N_α-Cbz-β-amino-L-alanine (**4**) was synthesized according to literature.[34] All experiments were run in oven-dried glassware. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance 400 spectrometer. The chemical shifts are reported relative to the deuteriated solvents. Peak assignments are based on either DEPT and/or comparison with literature data. The ESI and HR mass spectra were recorded with a Finnigan MAT 900 S spectrometer. Melting points are not corrected. Purity of compounds was determined using analytical HPLC with a Dionex HPLC apparatus consisting of a P680 HPLC pump, an ASI-100 automated sample injector, and a UVD 340U UV detector with a Supelcosil LC-18 (25 cm × 4.6 mm, 5 μm) column. Commercially available HPLC grade solvents were used as eluents. All compounds used for solution phase assays had a purity of > 95%.

Mono-*tert*-butyl Fumarate (2). A solution of oxalyl chloride (24.0 g, 189 mmol) in dichloromethane (20 mL) was added dropwise to a solution of fumaric acid (**1**, 20.0 g, 172 mmol) in dichloromethane (100 mL) and DMF (5 mL). The mixture was stirred for 1 h at room temperature, and the solvent was removed by distillation under reduced pressure. The remaining residue was dissolved in *tert*-butyl alcohol (75 mL). The solution was cooled to 0 °C in an ice bath, and potassium *tert*-butylate (21.2 g, 189 mmol) was added. The mixture was stirred for 2 h at room temperature, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (hexane/ethyl acetate, 0.1% acetic acid) to give **2** (3.40 g, 12%) as a white solid: mp 66 °C. ^{1}H NMR (400 MHz, DMSO-d_6, 27 °C): δ = 1.46 (s, 9H, CH_3), 6.59 (dd, $^3J_{HH}$ = 15.6 Hz, $^4J_{HH}$ = 19.8 Hz, 2H, 2 × olefin CH), 13.08 (br s, 1H, COOH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6, 27 °C): δ = 27.6 (CH_3), 81.4 (C_qMe_3), 133.8, 134.3 (olefin CH), 163.7, 165.8 (C=O) ppm. FT-IR (KBr pellet): $\tilde{\nu}$ = 2985 (m), 1714 (s), 1472 (w), 1423 (w), 1370 (m), 1305 (m), 1272 (s), 1160 (s) cm^{-1}. HR-MS (positive ESI): calcd for $C_8H_{11}O_4$ [M − H$^+$] 171.06628; found 171.06623.

N_α-Cbz-β-acetylamino-L-alanine (5). Acetic anhydride (3.60 mL, 37.4 mmol) was added dropwise to a suspension of N_α-Cbz-β-amino-L-alanine (**4**, 8.10 g, 34.0 mmol) in dichloromethane (200 mL). Then triethylamine (10.5 mL, 74.8 mmol) was added, and the reaction mixture was stirred for 5 h at room temperature until the suspension turned into a clear solution. The solvent was removed under reduced pressure. The obtained oil was suspended in water and lyophilized to give **5** (8.01 g, 84%) as a white solid: mp 43 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6, 27 °C): δ = 1.77 (s, 3H, CH_3), 3.23−3.37 (m, 2H, CH_2), 3.84−3.89 (m, 1H, CH), 5.01 (s, 2 H, Cbz-CH_2), 6.90 (d, 1H, $^3J_{HH}$ = 7.4 Hz, NHCbz), 7.29−7.38 (m, 5H, aryl CH), 7.79 (br s, 1H, NH) ppm. ^{13}C NMR (100 MHz,]DMSO-d_6, 27 °C): δ = 22.5 (CH_3), 39.8 (CH_2NH), 54.0 (CH), 65.5 (Cbz-CH_2), 127.7, 127.8, 128.3 (aryl CH), 136.9 (Cq), 156.0, 169.9, 172.0 (C=O) ppm. FT-IR (KBr pellet): $\tilde{\nu}$ = 3350 (m), 3060 (w), 2950 (w), 1720 (s), 1540 (s), 1225 (m), 1065 (m) cm^{-1}. HR-MS (negative ESI): calcd for $C_{13}H_{15}N_2O_5$ [M − H$^+$] 279.09865; found 279.09864.

N_α-Fmoc-β-acetylamino-L-alanine (7). A mixture of **5** (8.01 g, 28.6 mmol) and Pd/C (801 mg) in methanol (150 mL) was hydrogenated at 35 °C for 7 h. The mixture was filtered through Celite to remove Pd/C. The Celite was washed with a mixture of 5% hydrochloric acid and methanol (1/1, 100 mL), and the solvent of the combined filtrates was evaporated to give the deprotected amino acid (6.34 g) as a white solid. This crude product was used in the next step without further purification.

A solution of Fmoc-Cl (8.14 g, 31.5 mmol) in dioxane was added dropwise at 0 °C to a solution of the crude amino acid **6** (6.34 g) in an aqueous solution of sodium carbonate (100 mL, 10%). The reaction mixture was stirred at room temperature for 20 h and then acidified with concentrated hydrochloric acid (pH 2). The mixture was extracted with dichloromethane (3 × 100 mL). The solvent of the combined organic layers was evaporated to obtain a yellow oil. The crude product was purified by flash column chromatography on silica gel (dichloromethane/methanol = 9/1, 0.1% acetic acid) to give **7** (3.45 g, 33%) as a white solid: mp 237 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6, 27 °C): δ = 1.80 (s, 3H, CH_3), 3.27−3.34 (m, 1H, CH_2), 3.44−3.48 (m, 1H, CH_2), 4.04−4.09 (m, 1H, CH), 4.23−4.30 (m, 3H, Fmoc-CH_2, Fmoc-CH), 7.33 (t, $^3J_{HH}$ = 7.3 Hz, 2H, Fmoc-aryl CH), 7.42 (m, 3H, Fmoc-aryl CH, Fmoc-NH), 7.72 (d, $^3J_{HH}$ = 7.4 Hz, 2H, Fmoc-aryl CH), 7.90 (m, 3H, Fmoc-aryl CH, CH_2NH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6, 27 °C): δ = 22.5 (CH_3), 39.8 (CH_2NH_2), 46.6 (Fmoc-CH), 54.1 (CH), 65.7 (Fmoc-CH_2), 120.1, 125.2, 127.1, 127.6 (Fmoc-aryl CH), 140.7, 143.8 (Fmoc-aryl Cq), 155.9, 169.7, 172.1 (C=O) ppm. FT-IR (KBr pellet): $\tilde{\nu}$ = 3405 (m), 2931 (w), 1700 (s), 1660 (s), 1545 (s), 1265 (m), 1035 (m) cm^{-1}. HR-MS (positive ESI): calcd for $C_{20}H_{20}N_2O_5$ [M + Na$^+$] 391.12644; found 391.12575.

Solid-Phase Synthesis of the Peptidic Inhibitors 8−21. The peptidic inhibitors were synthesized on MBHA resin according to a standard protocol. MBHA resin (300 mg, 1.3 mmol/g, 390 μmol) was swollen in DMF for 1 h, and the first four amino acids were coupled to the resin by the use of Fmoc-protected amino acids (2.5 equiv), HCTU (2.5 equiv), and *N*-methylmorpholine (NMM, 300 μL) in DMF (10 mL) for 20 h. Each coupling step was repeated twice, and the resin was washed with DMF (3 × 10 mL). The Fmoc group was then cleaved with piperidine in DMF (20%, 10 mL), and the resin was washed with DMF (6 × 10 mL). The fumarate **2** was introduced under the same coupling conditions using HCTU, NMM, and DMF. The *tert*-butyl ester was removed by the following conditions: the resin was washed with dichloromethane (3 × 10 mL), treated for 5 min with a solution of TFA in dichloromethane (25%, 10 mL), and treated for 30 min with a solution of TFA in dichloromethane (50%, 10 mL). The resin was then washed with dichloromethane (3 × 10 mL) and with DMF (3 × 10 mL). The next three amino acids were coupled using the *tert*-butyl ester protected amino acid (2.5 equiv), HCTU (2.5 equiv), NMM (300 μL), and DMF (10 mL) for 20 h. Each coupling step was repeated twice, the *tert*-butyl ester was removed as described above, and the resin was washed with dichloromethane (3 × 10 mL), methanol (3 × 10 mL), diethyl ether (2 × 10 mL), and dichloromethane (3 × 10 mL). Cleavage from the resin was performed by exposure to TFA/TFMSA (9/1) for 3 h. The resin was washed with TFA (3 × 10 mL), and the solvent of the combined filtrates was then evaporated to obtain a yellow oil. The residue was treated with hydrochloric acid (0.1 M, 20 mL), and the white precipitated solid was collected. In order to obtain a better solubility water and triethylamine (20%) were added, and the suspension was lyophilized. The ratio of contained triethylamine was different for each compound and shown in parentheses after the molecular formula. The amounts of triethylamine were determined by the integrals in the ^{1}H NMR spectra of the inhibitors. In some cases we observed the formation of triethylammonium chloride that could not be removed. The additional salt did not have any influence on the inhibition of the cysteine proteases.

Detailed spectroscopic data of these inhibitors can be found in the Supporting Information.

NH_2-Val←Leu←Leu←Asn←Fum-Gly-Gly-Phe-OH (8, 8/9 NEt_3). $C_{38}H_{57}N_9O_{11}$ (8/9 $C_6H_{15}N$): 905.86 g/mol. Yield 265 mg (293 μmol, 75%). Mp 228 °C (dec). MS (negative MALDI): calcd for $C_{38}H_{56}N_9O_{11}^-$ [M − H^+] 814.411; found 814.629.

NH_2-Val←Ala←Leu←Asn←Fum-Gly-Gly-Phe-OH (9, NEt_3, 1/2 $HNEt_3Cl$). $C_{35}H_{51}N_9O_{11}$ ($C_6H_{15}N$, 1/2 $C_6H_{15}ClN$): 1034.91 g/mol. Yield 266 mg (257 μmol, 66%). Mp 193 °C (dec). MS (negative MALDI): calcd for $C_{35}H_{50}N_9O_{11}^-$ [M − H^+] 772.364; found 772.362.

NH_2-Val←Leu←Phe←Asn←Fum-Gly-Gly-Phe-OH (10, 7/9 NEt_3). $C_{41}H_{55}N_9O_{11}$ (7/9 $C_6H_{15}N$): 928.63 g/mol. Yield 293 mg (316 μmol, 81%). Mp 165 °C (dec). MS (negative MALDI): calcd for $C_{41}H_{54}N_9O_{11}^-$ [M − H^+] 848.395; found 848.733.

NH_2-Val←Leu←Phg←Asn←Fum-Gly-Gly-Phe-OH (11, NEt_3). $C_{40}H_{54}N_9O_{11}$ ($C_6H_{15}N$): 937.09 g/mol. Yield 314 mg (335 μmol, 86%). Mp 205 °C (dec). MS (negative MALDI): calcd for $C_{40}H_{53}N_9O_{11}^-$ (M − H^+) 834.379; found 834.382.

NH_2-Val←Leu←Leu←Phe←Fum-Gly-Gly-Phe-OH (12, 8/9 NEt_3). $C_{43}H_{60}N_8O_{10}$ (8/9 $C_6H_{15}N$): 938.93 g/mol. Yield 308 mg (328 μmol, 84%). Mp 160 °C (dec). MS (negative MALDI): calcd for $C_{43}H_{59}N_8O_{10}^-$ [M − H^+] 847.436; found 847.796.

NH_2-Val←Val←Leu←Gln←Fum-Ala-Gly-Phe-OH (13, 2/3 NEt_3). $C_{39}H_{60}N_9O_{11}$ (2/3 $C_6H_{15}N$): 897.40 g/mol. Yield 311 mg (347 μmol, 89%). Mp 240 °C (dec). MS (negative MALDI): calcd for $C_{39}H_{59}N_9O_{11}^-$ [M − H^+] 828.426; found 828.751.

NH_2-Val←Ala←Chg←Asn←Fum-Gly-Gly-Phe-OH (14, NEt_3, 1/3 $HNEt_3Cl$). $C_{43}H_{68}N_{10}O_{11}$ (1/3 $C_6H_{15}N$): 946.94 g/mol. Yield 262 mg (277 μmol, 71%). Mp 245 °C (dec). MS (negative MALDI): calcd for $C_{43}H_{67}N_{10}O_{11}^-$ (M − H^+) 798.379; found 798.733.

NH_2-Val←Val←Chg←Asn←Fum-Gly-Gly-Phe-OH (15, NEt_3). $C_{39}H_{57}N_9O_{11}$ ($C_6H_{15}N$): 929.11 g/mol. Yield 250 mg (269 μmol, 69%). Mp 232 °C (dec). MS (negative MALDI): calcd for $C_{39}H_{56}N_9O_{11}^-$ [M − H^+] 826.411; found 826.661.

NH_2-Val←Val←Chg←Gln←Fum-Gly-Gly-Phe-OH (16, NEt_3, 2/3 $HNEt_3Cl$). $C_{40}H_{59}N_9O_{11}$ ($C_6H_{15}N$, 2/3 $C_6H_{16}ClN$): 1034.91 g/mol. Yield 319 mg (308 μmol, 79%). Mp 250 °C (dec). MS (negative MALDI): calcd for $C_{40}H_{58}N_9O_{11}^-$ [M − H^+] 840.426; found 840.661.

NH_2-Val←Ala←Chg←C1(Ac)←Fum-Gly-Gly-Phe-OH (17, NEt_3, 4/9 $HNEt_3Cl$). $C_{38}H_{55}N_9O_{11}$ ($C_6H_{15}N$, 4/9 $C_6H_{15}ClN$): 976.27 g/mol. Yield 263 mg (269 mmol, 69%). Mp 225 °C (dec). MS (negative MALDI): calcd for $C_{38}H_{54}N_9O_{11}^-$ [M − H^+] 812.395; found 812.660.

NH_2-Val←Leu←Leu←Ala←Fum-Gly-Gly-Phe-OH (18, 2/3-NEt_3). $C_{37}H_{56}N_8O_{10}$ (2/3 $C_6H_{15}N$): 840.35 g/mol. Yield 161 mg (191 μmol, 49%). Mp 244 °C (dec). MS (negative MALDI): calcd for $C_{37}H_{55}N_8O_{10}^-$ [M − H^+] 771.400; found 771.432.

NH_2-Val←Leu←Leu←Ala←Fum-Ala-Gly-Phe-OH (19, 7/9 NEt_3). $C_{38}H_{58}N_8O_{10}$ (7/9 $C_6H_{15}N$): 540.65 g/mol. Yield 287 mg (332 μmol, 85%). Mp 228 °C (dec). MS (negative MALDI): calcd for $C_{38}H_{57}N_8O_{10}^-$ [M − H^+] 785.420; found 785.421.

NH_2-Val←Leu←Leu←C1(Ac)←Fum-Gly-Gly-Phe-OH (20, 8/9 NEt_3). $C_{39}H_{59}N_9O_{11}$ (8/9 $C_6H_{15}N$): 919.89 g/mol. Yield 190 mg (207 μmol, 53%). Mp 231 °C (dec). MS (negative MALDI): calcd for $C_{39}H_{58}N_9O_{11}^-$ [M − H^+] 828.430; found 828.408.

NH_2-Val←Ala←Leu←C1(Ac)←Fum-Gly-Gly-Phe-OH (21, 8/9 NEt_3). $C_{36}H_{53}N_9O_{11}$ (8/9 $C_6H_{15}N$): 877.81 g/mol. Yield 277 mg (316 μmol, 81%). Mp 239 °C (dec). MS (negative MALDI): calcd for $C_{36}H_{52}N_9O_{11}^-$ [M − H^+] 786.380; found 786.366.

Protease Inhibition. General. Cathepsin B (human liver) was purchased from Calbiochem and cathepsin L (bovine spleen) from Merck; falcipain-2 and rhodesain were recombinantly expressed as described previously (FP-2[48] and RD[54]). Substrates were purchased from Bachem. Assay buffer: falcipain-2 and rhodesain, 50 mM acetate buffer, pH 5.5, 5 mM EDTA, 200 mM NaCl, 5 mM DTT; cathepsins B and L, 50 mM Tris buffer, pH 6.5, 2 mM DTT, 5 mM EDTA, 200 mM NaCl. Substrates and inhibitor stock solutions were prepared in DMSO and diluted with the respective assay buffer (final DMSO concentration: 10%). Total volume: 250 μL (215 μL of assay buffer, 10 μL of enzyme solution, 20 μL of DMSO or inhibitor solution, 5 μL of substrate solution). Enzyme stock solutions were diluted with assay buffer to yield a fluorescence increase of dF/min ≈ 20. A Varian Cary eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) with 96-well plates was used; λ_{ex} = 380 nm and λ_{em} = 460 nm.

Screening. The hydrolysis of the substrate Cbz-Phe-Arg-AMC (40 μM) or Cbz-Leu-Arg-AMC (in the case of **12** and **13**, 25 μM) by the respective enzyme was monitored in the absence or presence of 100 μM inhibitor (final concentration). The slope of the progress curve in the absence of inhibitor was set to 100% ($= v_c$). Percentage of inhibition was calculated using the equation % inhibition = $100(1 - v_i/v_c)$ with v_i as the inhibited rate of the reaction.

Determination of K_i Values. The hydrolyses of the substrates were monitored over a period of 5−10 min in the presence of 0−80 μM inhibitor (final inhibitor concentrations: 0.1, 1, 2, 4, 5, 6, 8, 10, 20, 40, 50, 60, 80 μM). The K_i values were calculated using the Dixon equation $v_o/v_i = 1 + ([I]/K_i^{app})$ and correction to zero substrate concentration with $K_i = K_i^{app}/(1 + [S]/K_m)$ with [S] = 40 μM and K_m = 21.5 μM for falcipain-2, 827 nM for rhodesain, 150 μM for cathepsin B, and 6.25 and 6.5 μM for cathepsin L. The GraFit6 software was used to calculate the K_i^{app} values.

Evaluations of Cultured Malaria Parasites. These were done as described previously.[50]

Antitrypanosomal Activities. These were done as described previously.[55]

On-Bead Screening. The respective enzyme was incubated with samples of the resin-bound inhibitors. The resin was added in isopyknotic suspensions in DMSO (20 μL, 20 mg/mL). The hydrolysis of the substrate Cbz-Phe-Arg-AMC (40 μM) by the proteases was monitored in the presence of 0.64 mM inhibitor (final concentration, determined by the theoretical loading of the resin, 0.40 mmol/g). The obtained kinetic data were compared with a sample of amino-functionalized PEGA resin (without inhibitor). The slope of the progress curve of the PEGA resin was set to 100% so that the inhibition could be determined using the equation: % inhibition = $100(1 - v_i/v_c)$.

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Supporting Information Available: On-bead inhibition data of the complete combinatorial library for falcipain-2 (FP-2) and rhodesain (Rd) and spectroscopic data of peptidic inhibitors **8**−**21**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) WHO. *The World Health Report 2000*. World Health Organization: Geneva, 2000.
(2) Sternberg, J. M. Human African trypanosomiasis: clinical presentation and immune response. *Parasite Immunol*. **2004**, *26*, 469–476.
(3) Caffrey, C. R.; Scory, S.; Steverding, D. Cysteine proteinases of trypanosome parasites: novel targets for chemotherapy. *Curr. Drug Targets* **2000**, *1*, 155−162, and references cited therein.
(4) Snow, R. N.; Craig, M.; Deichmann, U.; Marsh, K. Estimating mortality, morbidity and disability due to malaria among Africa′s non-pregnant population. *Bull. W.H.O*. **1999**, *77*, 624–640. Dominguez, J. N. Chemotherapeutic agents against malaria: what next after chloroquine? *Curr. Trop. Med. Chem*. **2002**, *2*, 1173–1185.

(5) White, N. J. Drug resistance in malaria. *Br. Med. Bull.* **1998**, *54*, 703–715. Breman, J. G. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am. J. Trop. Med. Hyg.* **2001**, *64*, 1–11.
(6) Rosenthal, P. J. Cysteine proteases of malaria parasites. *Int. J. Parasitol.* **2004**, *34*, 1489–1499.
(7) Caffrey, C. R.; Hansell, E.; Lucas, K. D.; Brinen, L. S.; Hernandez, A. A.; Cheng, J.; Gwaltney, S. L., II; Roush, W. R.; Stierhof, Y. D.; Bogyo, M.; Steverding, D.; McKerrow, J. H. Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. *Mol. Biochem. Parasitol.* **2001**, *118*, 61–73.
(8) Shenai, B. R.; Sijwali, P. S.; Singh, A.; Rosenthal, P. J. Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*. *J. Biol. Chem.* **2000**, *275*, 29000–29010. Sabnis, Y. A.; Desai, P. V.; Rosenthal, P. J.; Avery, M. A. Probing the structure of falcipain-3, a cysteine protease from Plasmodium falciparum: comparative protein modeling and docking studies. *Protein Sci.* **2003**, *12*, 501–509.
(9) Rosenthal, P. J.; Sijwali, P. S.; Singh, A.; Shenai, B. R. Cysteine proteases of malaria parasites: targets for chemotherapy. *Curr. Pharm. Des.* **2002**, *8*, 1659–1672.
(10) Sijwali, P. S.; Rosenthal, P. J. Gene disruption confirms a critical role for the cysteine protease falcipain-2 in hemoglobin hydrolysis by *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4384–4389.
(11) Barrett, A. J. Bioinformatics of proteases in the MEROPS database. *Curr. Opin. Drug Discov. Dev.* **2004**, *7*, 334–341; http://merops.sanger.ac.uk/index.htm.
(12) Gelhaus, C.; Vicik, R.; Schirmeister, T.; Leippe, M. Blocking effect of a biotinylated protease inhibitor on the egress of *Plasmodium falciparum* merozoites from infected red blood cells. *Biol. Chem.* **2005**, *386*, 499–502. Francis, S. E.; Sullivan, D. J., Jr.; Goldberg, D. E. Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. *Annu. Rev. Microbiol.* **1997**, *51*, 97–123. McKerrow, J. H.; Sun, E.; Rosenthal, P. J.; Bouvier, J. The proteases and pathogenicity of parasitic protozoa. *Annu. Rev. Microbiol.* **1993**, *47*, 821–853.
(13) Eksi, S.; Czesny, B.; Greenbaum, D. C.; Bogyo, M.; Williamson, K. C. Targeted disruption of *Plasmodium falciparum* cysteine protease, falcipain 1, reduces oocyst production, not erythrocytic stage growth. *Mol. Microbiol.* **2004**, *53*, 243–250.
(14) Singh, A.; Rosenthal, P. J. Selection of cysteine protease inhibitor-resistant malaria parasites is accompanied by amplification of falcipain genes and alteration in inhibitor transport. *J. Biol. Chem.* **2004**, *34*, 35236–35241.
(15) Ramjee, M. K.; Flinn, N. S.; Pemberton, T. P.; Quibell, M.; Wang, Y.; Watts, J. P. Substrate mapping and inhibitor profiling of falcipain-2, falcipain-3 and berghepain-2: implications for peptidase anti-malarial drug discovery. *Biochem. J.* **2006**, *399*, 47–57.
(16) St. Hilaire, P. M.; Alves, L. C.; Sanderson, S. J.; Mottram, J. C.; Juliano, M. A.; Juliano, L.; Coombs, G. H.; Meldal, M. The substrate specificity of a recombinant cysteine protease from *Leishmania mexicana*: application of a combinatorial peptide library approach. *ChemBioChem* **2000**, *1*, 115–122.
(17) For a review see: Maly, D. J.; Huang, L.; Ellman, J. A. Combinatorial strategies for targeting protein families: application to the proteases. *ChemBioChem* **2002**, *3*, 16–37.
(18) Cuerrier, D.; Moldoveanu, T.; Campbell, R. L.; Kelly, J.; Yoruk, B.; Verhelst, S. H. L.; Greenbaum, D.; Bogyo, M.; Davies, P. L. Development of calpain-specific inactivators by screening of positional scanning epoxide libraries. *J. Biol. Chem.* **2007**, *282*, 9600–9611.
(19) Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J.* **1982**, *201*, 189–198.
(20) Darkins, P.; Gilmore, B. F.; Hawthorne, S. J.; Healy, A.; Moncrieff, H.; McCarthy, N.; McKervey, M. A.; Bromme, D.; Papagano, M.; Walker, B. Synthesis of peptidyl ene diones: selective inactivators of the cysteine proteinases. *Chem. Biol. Drug Des.* **2007**, *69*, 170–179.
(21) Ekici, Ö. D.; Gotz, M. G.; James, K. E.; Li, Z. Z.; Rukamp, B. J.; Asgian, J. L.; Caffrey, C. R.; Hansell, E.; Dvořák, J.; McKerrow, J. H.; Potempa, J.; Travis, J.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. Aza-peptide Michael acceptors: a new class of inhibitors specific for caspases and other clan CD cysteine proteases. *J. Med. Chem.* **2004**, *47*, 1889–1892.
(22) Schirmeister, T. Aziridine-2,3-dicarboxylic acid derivatives as inhibitors of papain. *Arch. Pharm. Pharm. Med. Chem.* **1996**, *329*, 229–238.
(23) Santos, M. M. M.; Moreira, R. Michael acceptors as cysteine protease inhibitors. *Mini-Rev. Med. Chem.* **2007**, *7*, 1040–1050.
(24) Matthews, D. A.; Dragovich, P. S.; Webber, S. E.; Fuhrman, S. A.; Patick, A. K.; Zalman, L. S.; Hendrickson, T. F.; Love, R. A.; Prins, T. J.; Marakovits, J. T.; Zhou, R.; Tikhe, J.; Ford, C. E.; Meador, J. W.; Ferre, R. A.; Brwon, E. L.; Binford, S. L.; Brothers, M. A.; Delisle, D. M.; Worland, S. T. Structure-assisted design of mechanism-based irreversible inhibitors of human rhinovirus 3C protease with potent antiviral activity against multiple rhinovirus serotypes. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11000–11007.
(25) (a) Jung, G.; Beck-Sickinger, A. G. Methoden der multiplen Peptidsynthese und ihre Anwendungen. *Angew. Chem.* **1992**, *104*, 375–391. (b) Balkenhohl, F.; Bussche-Hunnefeld, C. v. d.; Lansky, A.; Zechel, C. Kombinatorische Synthese niedermolekularer organischer Verbindungen. *Angew. Chem.* **1996**, *108*, 2436–2488.
(26) Bayer, E. Towards the chemical synthesis of proteins. *Angew. Chem., Int. Ed.* **1991**, *30*, 113–129.
(27) Meldal, M. PEGA: A flow stable polyethylene glycol dimethyl acrylamide copolymer for solid phase synthesis. *Tetrahedron Lett.* **1992**, *33*, 3077–3080. Auzanneau, F.-I.; Meldal, M.; Bock, K. Synthesis, characterization and biocompatibility of PEGA resins. *J. Pept. Sci.* **1995**, *1*, 31–44. Renil, M.; Ferreras, M.; Delaissé, J. M.; Foged, N. T.; Meldal, M. PEGA supports for combinatorial peptide synthesis and solid-phase enzymatic library assays. *J. Pept. Sci.* **1998**, *4*, 195–210.
(28) Grotli, M.; Gotfredsen, C. H.; Rademann, J.; Burchard, J.; Clark, A. J.; Duus, J. O.; Meldal, M. Physical properties of poly(ethylene glycol) (PEG)-based resins for combinatorial solid phase organic chemistry: a comparison of PEG-cross-linked and PEG-grafted resins. *J. Comb. Chem.* **2000**, *2*, 108–119.
(29) Kim, D.-H.; Lee, H.-Y.; Kim, H.; Kim, H.; Lee, Y.-S.; Park, S. B. Quantitative evaluation of HiCore resin for the nonspecific binding of proteins by on-bead colorimetric assay. *J. Comb. Chem.* **2006**, *8*, 280–285.
(30) Xiao, X.-Y.; Parandoosh, Z.; Nova, M. P. Design and synthesis of a taxoid library using radiofrequency encoded combinatorial chemistry. *J. Org. Chem.* **1997**, *62*, 6029–6033. Nicolaou, K. C.; Pfefferkorn, J. A.; Mitchell, H. J.; Roecker, A. J.; Barluenga, S.; Cao, G.-Q.; Affleck, R. L.; Lillig, J. E. Natural product-like combinatorial libraries based on privileged structures. 2. Construction of a 10 000-membered benzopyran library by directed split-and-pool chemistry using NanoKans and optical encoding. *J. Am. Chem. Soc.* **2000**, *122*, 9954–9967.
(31) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595–598.
(32) Attardi, M. E.; Porcu, G.; Taddei, M. Malachite green, a valuable reagent to monitor the presence of free COOH on the solid-phase. *Tetrahedron Lett.* **2000**, *41*, 7391–7394.
(33) Gutheil, W. G.; Xu, Q. N-to-C solid-phase peptide and peptide trifluoromethylketone synthesis using amino acid tert-butyl esters. *Chem. Pharm. Bull.* **2002**, *50*, 688–691.
(34) Zhang, L.; Kauffman, G. S.; Pesti, J. A.; Yin, J. Rearrangement of Nα-protected L-asparagines with iodosobenzene diacetate. A practical route to β-amino-L-alanine derivatives. *J. Org. Chem.* **1997**, *62*, 6918–6920.
(35) Smith, H. K.; Bradley, M. Comparison of resin and solution screening methodologies in combinatorial chemistry and the identification of a 100 nM inhibitor of trypanothione reductase. *J. Comb. Chem.* **1999**, *1*, 326–332.
(36) Robins, L. I.; Dixon, S. M.; Wilson, D. K.; Kurth, M. J. On-bead combinatorial techniques for the identification of selective aldose reductase inhibitors. *Bioorg. Med. Chem.* **2006**, *14*, 7728–7735.
(37) Dixon, S. M.; Li, P.; Liu, R.; Wolosker, H.; Lam, K. S.; Kurth, M. J.; Toney, M. D. Slow-binding human serine racemase inhibitors from high-throughput screening of combinatorial libraries. *J. Med. Chem.* **2006**, *49*, 2388–2397.
(38) Spetzler, J. C.; Westphal, V.; Winther, J. R.; Meldal, M. Preparation of fluorescence quenched libraries containing interchain disulphide bonds for studies of protein disulphide isomerases. *J. Pept. Sci.* **1998**, *4*, 128–137.
(39) Shin, D.-S.; Kim, Y.-G.; Kim, E.-M.; Kim, M.; Park, H.-Y.; Kim, J.-H.; Lee, B.-S.; Kim, B.-G.; Lee, Y.-S. Solid-phase peptide library synthesis on HiCore resin for screening substrate specificity of Brk protein tyrosine kinase. *J. Comb. Chem.* **2008**, *10*, 20–23.
(40) For substrate libraries see: (a) Leon, S.; Quarrell, R.; Lowe, G. Evaluation of resins for on-bead screening: A study of papain and chymotrypsin specificity using PEGA-bound combinatorial peptide libraries. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2997–3002. (b) St. Hilaire, P. M.; Willert, M.; Juliano, M. A.; Juliano, L.; Meldal, M. Fluorescence-quenched solid phase combinatorial libraries in the characterization of cysteine protease substrate specificity. *J. Comb. Chem.* **1999**, *1*, 509–523. (c) Rossé, G.; Kueng, E.; Page, M. G. P.;

Schauer-Vukasinovic, V.; Giller, T.; Lahm, H.-W.; Hunziker, P.; Schlatter, D. Rapid identification of substrates for novel proteases using a combinatorial peptide library. *J. Comb. Chem.* **2000**, *2*, 461–466. (d) Doezé, R. H. P.; Maltman, B. A.; Egan, C. L.; Ulijn, R. V.; Flitsch, S. L. Profiling primary protease specificity by peptide synthesis on a solid support. *Angew. Chem., Int. Ed.* **2004**, *43*, 3138–3141. (e) Alves, F. M.; Hirata, I. Y.; Gouvea, I. E.; Alves, M. F. M.; Meldal, M.; Bromme, D.; Juliano, L.; Juliano, M. A. Controlled peptide solvation in portion-mixing libraries of FRET peptides: Improved specificity determination for Dengue 2 virus NS2B-NS3 protease and human cathepsin S. *J. Comb. Chem.* **2007**, *9*, 627–634. (f) Kofoed, J.; Reymond, J.-L. Identification of protease substrates by combinatorial profiling on TentaGel beads. *Chem. Commun.* **2007**, 4453–4455. (g) Deere, J.; McDonnell, G.; Lalaouni, A.; Maltman, B. A.; Flitsch, S. L.; , Halling, P. J. Real time imaging of protease action on substrates covalently immobilised to polymer supports. *Adv. Synth. Catal.* **2007**, *349*, 1321−1326. For inhibitor libraries check: (h) Meldal, M.; Svendsen, I. Direct visualization of enzyme inhibitors using a portion mixing inhibitor library containing a quenched fluorogenic peptide substrate. Part 1. Inhibitors for subtilisin Carlsberg. *J. Chem. Soc., Perkin Trans. 1* **1995**, 1591–1596. (i) Meldal, M.; Svendsen, I.; Juliano, L.; Juliano, M. A.; Del Nery, E.; Scharfstein, J. Inhibition of cruzipain visualized in a fluorescence quenched solid-phase inhibitor library assay. D-Amino acid inhibitors for cruzipain, cathepsin B and cathepsin L. *J. Pept. Sci.* **1998**, *4*, 83–91. (j) Burchard, J.; Schiodt, C. B.; Krog-Jensen, C.; Delaissé, J.-M.; Foged, N. T.; Meldal, M. Solid phase combinatorial library of phosphinic peptides for discovery of matrix metalloproteinase inhibitors. *J. Comb. Chem.* **2000**, *2*, 624–638. (k) Graven, A.; St. Hilaire, P. M.; Sanderson, S. J.; Mottram, J. C.; Coombs, G. H.; Meldal, M. Combinatorial library of peptide isosters based on Diels-Alder reactions: identification of novel inhibitors against a recombinant cysteine protease from Leishmania mexicana. *J. Comb. Chem.* **2001**, *3*, 441–452. (l) St. Hilaire, P. M.; Alves, L. C.; Herrera, F.; Renil, M.; Sanderson, S. J.; Mottram, J. C.; Coombs, G. H.; Juliano, M. A.; Juliano, L.; Arevalo, J.; Meldal, M. Solid-phase library synthesis, screening, and selection of tight-binding reduced peptide bond inhibitors of a recombinant Leishmania mexicana cysteine protease B. *J. Med. Chem.* **2002**, *45*, 1971–1982. (m) Tornoe, C. W.; Sanderson, S. J.; Mottram, J. C.; Coombs, G. H.; Meldal, M. Combinatorial library of peptidotriazoles: identification of [1,2,3]-triazole inhibitors against a recombinant Leishmania mexicana cysteine protease. *J. Comb. Chem.* **2004**, *6*, 312–324.

(41) Meldal, M. Smart combinatorial assays for the determination of protease activity and inhibition. *QSAR Comb. Sci.* **2005**, *24*, 1141–1148.

(42) Rawlings, N. D.; Morton, F. R.; Kok, C. Y.; Kong, J.; Barrett, A. J. *MEROPS*: the peptidase database. *Nucleic Acids Res.* **2008**, *36*, D320–D325.

(43) Rosenthal, P. J. Falcipains. In *Handbook of Proteolytic Enzymes*, 2nd ed.; Barrett, A. J., Rawlings, N. D., Woessner, J. F., Eds.; Elsevier: London, 2004; pp 1157−1160.

(44) Yang, H.; Xie, W.; Xue, X.; Yang, K.; Ma, J.; Liang, W.; Zhao, Q.; Zhou, Z.; Pei, D.; Ziebuhr, J.; Hilgenfeld, R.; Yuen, K. Y.; Wong, L.; Gao, G.; Chen, S.; Chen, Z.; Ma, D.; Bartlam, M.; Rao, Z. Design of wide-spectrum inhibitors targeting coronavirus main proteases. *PLoS Biol.* **2005**, *3*, 1742–1752.

(45) Schulz, F.; Gelhaus, C.; Degel, B.; Vicik, R.; Heppner, S.; Breuning, A.; Leippe, M.; Gut, J.; Rosenthal, P. J.; Schirmeister, T. Screening of protease inhibitors as antiplasmodial agents. Part I: aziridines and epoxides. *ChemMedChem* **2007**, *2*, 1214–1224.

(46) Vicik, R.; Hoerr, V.; Glaser, M.; Schultheis, M.; Hansell, E.; McKerrow, J. H.; Holzgrabe, U.; Caffrey, C. R.; Ponte-Sucre, A.; Moll, H.; Stich, A.; Schirmeister, T. Aziridine-2,3-dicarboxylate inhibitors targeting the major cysteine protease of *Trypanosoma brucei* as lead trypanocidal agents. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2753–2757.

(47) Desai, P. V.; Patny, A.; Gut, J.; Rosenthal, P. J.; Tekwani, B.; Srivastava, A.; Avery, M. Identification of novel parasitic cysteine protease inhibitors by use of virtual screening. 2. The available chemical directory. *J. Med. Chem.* **2006**, *49*, 1576–1584.

(48) Pandey, K. C.; Wang, S. X.; Sijwali, P. S.; Lau, A. L.; McKerrow, J. H.; Rosenthal, P. J. The *Plasmodium falciparum* cysteine protease falcipain-2 captures its substrate, hemoglobin, via a unique motif. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9138–9143.

(49) In case of irreversible inhibitors, both the dissociation constant (K_i) of the noncovalent enzyme−inhibitor complex (EI) as a parameter for the first inhibition step and the alkylation rate (k_i) as a parameter of the second irreversible inhibition step leading to the covalently modified enzyme (E−I) are normally used to characterize inhibition potency. Since in the present cases saturation conditions could not be reached, only the approximated second-order rate constants of inhibition k_{second}^{app} ($k_{second} = k_i/K_i \approx k_{second}^{app} = k_{obs}/[I](1 + [S]/K_m)$) but not the single inhibition constants k_i and K_i could be determined in dilution assays. Therefore, inhibitors can only be compared concerning their overall inhibition potency but not concerning their affinities represented by K_i. Since all inhibitors contain the same electrophilic moiety, namely, fumaric acid amide, the alkylation rates (k_i) should be quite similar. Thus, we decided to determine the dissociation constants K_i in continuous assays without preincubation of enzymes and inhibitors measuring fluorescence increase over a period of 5−10 min. Within this measurement period progress curves turned out to be linear, thus representing the first noncovalent inhibition step which is characterized by the dissociation constant K_i of the noncovalent enzyme−inhibitor complex.

(50) Evers, A.; Heppner, S.; Leippe, M.; Gelhaus, C. An efficient fluorimetric method to measure the viability of intraerythrocytic *Plasmodium falciparum*. *Biol Chem.* **2008**, *389*, 1523–1525.

(51) Musonda, C. C.; Gut, J.; Rosenthal, P. J.; Yardley, V.; Carvalho de Souza, R. C.; Chibale, K. Application of multicomponent reactions to antimalarial drug discovery. Part 2: new antiplasmodial and antitrypanosomal 4-aminoquinoline gamma- and delta-lactams via a "catch and release" protocol. *Bioorg. Med. Chem.* **2006**, *14*, 5605–5615.

(52) Sijwali, P. S.; Koo, J.; Singh, N.; Rosenthal, P. J. Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **2006**, *150*, 96–106.

(53) Feng, B. Y; Shoichet, B. K. A detergent-based assay for the detection of promiscuous inhibitors. *Nat. Protoc.* **2006**, *1*, 550–553.

(54) Caffrey, C. R.; Hansell, E.; Lucas, K. D.; Brinen, L. S.; Hernandez, A. A.; Cheng, J.; Gwaltney, S. L., II; Roush, W. R.; Stierhof, Y. D.; Bogyo, M.; Steverding, D.; McKerrow, J. H. Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. *Mol. Biochem. Parasitol.* **2001**, *118*, 61–73.

(55) Vicik, R.; Hoerr, V.; Glaser, M.; Schultheis, M.; Hansell, E.; McKerrow, J. H.; Holzgrabe, U.; Caffrey, C. R.; Ponte-Sucre, A.; Moll, H.; Stich, A.; Schirmeister, T. Aziridine-2,3-dicarboxylate inhibitors targeting the major cysteine protease of *Trypanosoma brucei* as lead trypanocidal agents. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2753–2757.