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Inhibition of Cruzipain Visualized in a Fluorescence Quenched Solid-phase Inhibitor Library Assay. D-Amino Acid Inhibitors for Cruzipain, Cathepsin B and Cathepsin L

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Abstract: A PEGA-resin was derivatized with a 3 : 1 mixture of hydroxymethyl benzoic acid and Fmoc-Lys(Boc)-OH and the fluorogenic substrate Ac-Y(NO_2)KLRFSKQK(Abz)–PEGA was assembled on the lysine using the active ester approach. Following esterification of the hydroxymethyl benzoic acid with Fmoc-Val-OH a library XXX-k/r-XXXV containing approximately 200,000 beads was assembled by split synthesis. The resulting 'one bead, two peptides' library was subjected to extensive hydrolysis with cruzipain. One hundred darker beads were isolated and the 14 most persistently dark beads were collected and sequenced. The putative inhibitor peptides and several analogues were synthesized and found to be competitive μM to nM inhibitors of cruzipain in solution. The inhibitory activity was found to be unspecific to cruzipain when compared with cathepsins B and L and specific when compared with kallikrein. One of the inhibitors was docked into the active site of cathepsin B and was found most probably to bind to the enzyme cavity in an unusual manner, owing to the inserted D-amino acid residue. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: fluorescence quenched assay; inhibitor library; *Trypanosoma cruzi*; cathepsin B and L inhibitors; Parasitic protease inhibition

Abbreviations: AMC/MCA, aminomethyl coumarin; Abz, 2-aminobenzoic acid; Dhbt-OH, 3-hydroxy-3,4-didehydro-4-oxo-1,2,3-benzotriazine; HMBA, 4-hydroxymethyl benzoic acid; MCPS, multiple column peptide synthesis; MALDI-TOF MS, matrix-assisted laser desorption time of flight mass spectrometry; MSNT, mesitylenesulphon-1-yl-3-nitro-1,2,4-triazole; NEM, *N*-ethyl morpholine; PEGA, *bis*-aminopropyl ethylene glycol polyacrylamide copolymer; Pfp, pentafluorophenyl; TBTU, *O*-benzotriazo-1-yl-*N*,*N*,*N*,′*N*′-tetramethyl uronium tetrafluoroborate; three or one letter codes are used for the amino acids according to IUPAC. D-Amino acids are indicated by lower case lettering.

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Chargas' disease, the chronic disease caused by the intracellular protozoan *Trypanosoma cruzi*, is the single most important factor of chronic myocardial disease in South America [1]. Transmitted by blood-sucking triatomine insects or through contaminated blood supplies, and affecting over 20 million people [2], this highly debilitating chronic disease remains a major problem of public health. This is aggravated by the fact that currently available therapy is not always effective, and because of the treatment's toxicity, it is limited to short-term treatment of acute infections. The efforts to identify potential targets for drugs against this intracellular parasite have

recently converged towards a distinct class of proteolytic enzymes that are essential for parasite development: the cysteine proteinases from the papain superfamily [3–5]. Also known as cruzipains, cruzain or GP57/51, these proteases are encoded by a large number of closely related genes [6]. Characterized as a cathepsin L-like proteinase and predominantly localized to the parasite's endolysosomal vesicles [4], this enzyme is sensitive to membrane-permeable synthetic inhibitors, some of which have markedly impaired parasite infectivity *in vitro* [7, 8] and *in vivo* [9]. The subsite substrate specificities of cruzipain were extensively characterized and differences with respect to the closely related mammalian cathepsin L and cathepsin B were recently documented [10]. Notably, the crystal structure of the catalytic domain of cruzain, a recombinant protease expressed without the unique C-terminal extension, was recently resolved at high resolution [9]. Prompted by these advances, the development of inhibitors with increased selectivity for this parasite target is being intensively pursued. In other enzyme systems, the screening of specific inhibitors has required assays of large arrays of available or newly synthesized compounds; most often the best inhibitors are sought through an exhaustive design and synthesis process [11–16]. These screening procedures are often laborious and expensive, and a large quantity of the appropriate enzyme is required for each compound assayed.

In recent years several new methods of defining enzyme inhibitors by parallel multiple syntheses or by library methods have been described. Promising techniques are the positional scanning solution libraries [17], the split synthesis solid-phase assays [18] and assays performed on spatially adressable surfaces [19]. Highly specific inhibitors can be identified by another promising strategy, based on the combined use of a quenched fluorogenic substrate with a split synthesis library of putative inhibitors [20]. Such assays may be performed in a beaded and biocompatible polyethylene glycol cross-linked polyamide (PEGA) resin; the latter was designed for peptide synthesis [21] with an open structure that allows biologically active proteins to permeate into the interior of the beads [22]. The PEGA polymer has been used for enzymatic reactions with glycosyl transferase [23] and proteases [22, 24]. We have recently characterized the substrate specificity for cruzipain using a library of solid-phase bound fluorescence quenched compounds containing a C-terminal Lys(Abz) and an N-terminal 3-nitrotyrosine (Tyr(NO_2)) as an efficient donor/acceptor pair for intramolecular resonance energy transfer [25, 26]; illuminating beads were thus subjected to amino acid sequencing, yielding data on the subsite specificity of cruzipain [10]. In the present study we demonstrate that a cruzipain inhibitor assay can also be developed. In this procedure the volume of the bead can be regarded as a confined space, i.e. a local environment in each bead in which two different compounds compete for the same or two separate but interdependent binding sites on the protease. As shown here, this technique greatly facilitates the search for lead compounds for cruzipain inhibitors. The inhibitory activity of compounds identified by this method can be confirmed in solution assays, and quickly improved by substitution using multiple column peptide synthesis of the lead peptides and their analogues.

The inhibitory activities of protease inhibitors depend on two properties: binding affinity and stability towards hydrolysis. The inhibitor should exhibit a strong binding to the combining site of the enzyme in order to compete with the natural substrate of the enzyme. Stability towards hydrolysis can be achieved by introducing an element of inhibition to prevent processing and release of products. This can be a substrate analogue compound in which the otherwise sensitive bonds were substituted by an inert bond, e.g. carbon–carbon or retro inverso bonds in hydrolytic reactions or by altering the stereochemistry of the reaction center. The binding affinity should preferably be selective for the target enzyme as best achieved with larger inhibitor molecules with an increased number of polar, hydrophobic and electrostatic contacts to the active site residues of the enzyme. Increased binding can often be obtained with bonds resembling the transition state of the reaction. In the present work, a library of inhibitors containing D-amino acid as the element of inhibition has been generated.

In order to place two different peptides within the confined volume of a bead we temporarily shielded a fraction of the functional groups with hydroxymethyl benzoic acid and utilized the selectivity of active esters for amino nucleophiles to assemble the fluorescence quenched peptide substrate. After esterification of the hydroxyl function, the second peptide was synthesized as a library by split synthesis; a deprotected library could be generated, owing to the base labile and acid stable character of the ester bond to the hydroxymethyl benzamide [24].

The derivatization of the PEGA resin (Figure 1) was performed with TBTU and a mixture of 4-

hydroxymethyl benzoic acid (HMBA) and Fmoc-Lys(Boc)-OH. The Boc group was cleaved and the side chain derivatized with Boc-Abz-ODhbt. The cruzipain substrate Y(NO_2)KLRFSKQK(Abz) [10] was selected and synthesized using Fmoc-amino acid-OPfp esters. This was accomplished without noticeable acylation of the hydroxyl group reserved for the inhibitor synthesis. The N-terminal amino group of the substrate was acetylated with Ac-ODhbt to block the sequencing of the substrate in the subsequent sequence analysis. The esterification of the HMBA linker with Fmoc-Val-OH [27] was efficient as determined by amino acid analysis. The library was assembled in a simple custom-made library generator [28] with wet-mixing of the resin and parallel dispensing of solvents and deprotection reagents. The quality of the library was assessed by release from single beads and analysing by MALDI-TOF mass spectrometry.

In the present study, a $PEGA_{1900}$ resin with limited crosslinking was prepared by synthesis of a fluorogenic cruzipain-substrate containing Abz and Tyr(NO_2) and a library of peptides with variable sequence containing one D-amino acid. The resin was washed with NaOAc 25 mM, 2 mM EDTA pH 5.0 NaCl 0.2 M, DTT 2 mM and treated with 0.5 μM of cruzipain [29], corresponding to 50 fmol of enzyme used for each inhibitor tested. Substrate cleavage, albeit partial, was detected on highly illuminated beads, whereas darker beads showed no cleavage at all. Decreased fluorescence was observed in approximately 100 beads; the darker 14 beads among these were collected and sequenced by Edman degradation-gas phase sequencing, yielding the putative inhibitors listed in Table 1 and presented graphically in Figure 2. When sliced, the illuminated beads revealed a strong fluorescence along the periphery of the cut, indicating that the large cruzipain (a 57,000 Da glycoprotein) had only limited access to the interior portion of the resin beads. The analysis of the inhibitory compounds revealed that they were highly lipophilic, thus did not present a high incidence of positively charged residues as expected from a recent investigation of the substrate specificity [10]. None of the inhibitors showed any sign of proteolytic cleavage. A highly conserved consensus sequence (IWrYR/W) was found (Figure 3). The P_4 position was not occupied by any preferential residue. A strong preference for Ile was found for the P_3 position. Trp was preferred in subsite P_2, although aromatic amino acids such as Tyr were also observed. In spite of the equal representation of the D-amino acids lys and arg in the P_1 position of the library, only one of the inhibitory compounds found contained lys. This is also in contrast to the substrate studies, which did not reveal a preference of Lys over Arg in P_1 [10]. The P_1' sites preferentially contained Lys or Tyr, whereas P_2' and P_3' both showed some preference for Arg: P_2' was also frequently occupied by Trp.

The series of inhibitory peptides selected by the present solid-phase assay system (Table 1) as well as some analogues listed in Table 2 were synthesized by multiple column synthesis on high capacity $PEGA_{1900}$ resin [30], using a hydroxymethyl benzoic acid linker and esterification with Val (for 20

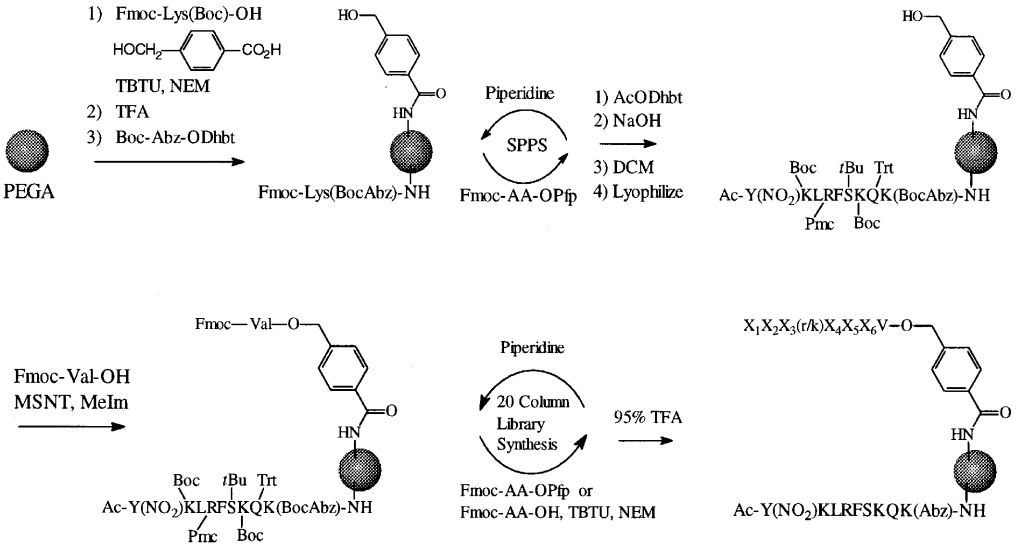

Figure 1 The strategy used in the synthesis of the 'one bead, two compounds' libraries.

Table 1 Inhibitors of Cruzipain Isolated from a Resin Bound Library XXX(r/k)XXXV–PEGA Containing the Substrate Ac-Y(NO_2)KLR-FSKQK(Abz), K_i for cruzipain was Measured Against Cbz-FR-Amc with $K_m = 1$ μM and $k_{cat} = 6\ s^{-1}$

Comp.	Inhibitor positions corresponding to enzyme subsites S4–S4′								K_I	MS on bead	Found	Calc.
	P4	P3	P2	P1	P′1	P′2	P′3	P′4	(μM)	M + H or Na	M + H	M
1	R	I	W	r	Y	W	A	V	0.11	1148.6	1149.3	1148.4
2	C	I	W	r	Y	W	R	V	0.25	1181.6	1181.4	1180.5
3	Y	F	F	r	M	F	G	V	0.36	1067.1	1066.2	1065.3
4	W	I	Y	r	W	F	F	V	0.36	1218.2	1216.3	1215.5
5	V	Y	W	r	R	R	Y	V	0.80	1193.1	1197.3	1196.4
6	T	I	W	r	K	L	G	V	1.77	972.3	971.9	971.2
7	E	I	W	r	Y	Y	W	V	1.77	1215.8	1214.3	1213.4
8	L	I	L	r	Y	W	T	V	2.64	1064.8	1063.4	1062.3
9	M	F	L	r	K	K	V	V	2.82	-ND	1020.8	1019.4
10	A	I	Y	r	Y	Y	F	V	4.53	1091.1	1094.3	1093.3
11	F	F	N	r	Y	I	R	V	7.00	1117.2	1114.3	1113.3
12	A	I	Y	r	Y	R	F	V	8.52	1091.1	1087.0	1086.3
13	G	F	Y	r	V	R	R	V	12.46	1053.4	1052.2	1051.3
14	V	I	F	r	K	L	A	V	37.58	939.5	917.8	916.2

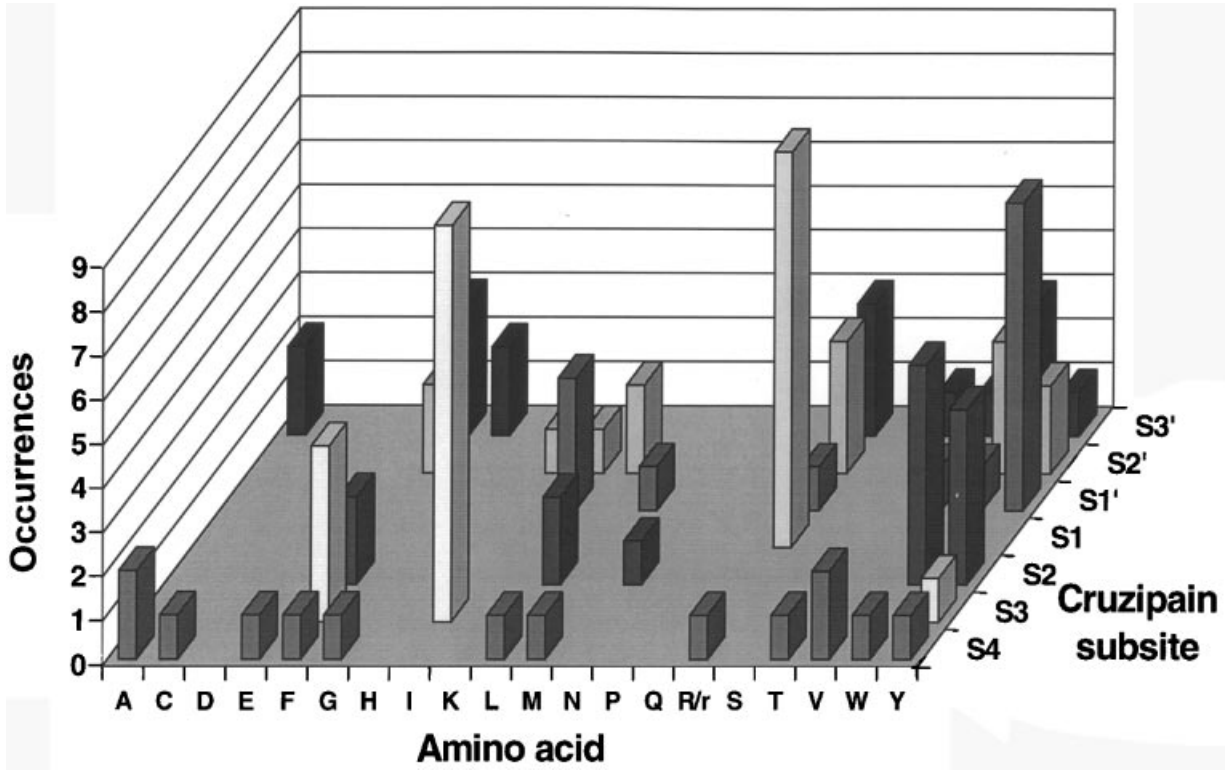

Figure 2 The distribution of amino acids found in 14 cruzipain inhibitors from a 'one bead, two compounds' library. The D-arg/lys residues have been assumed to be positioned in the P_1 subsite and the inhibitor is aligned in the direction of the substrate in accordance with MD calculations.

peptides) or val (for 7 peptides) [27]. The syntheses were performed in a manual 20 column synthesizer with two solvent delivery heads. The peptides were purified and analysed by HPLC, MS and amino acid analysis. The peptides were assayed as cruzipain inhibitors in solution. The inhibitors, all containing a D-amino acid, were completely stable to a 10^6 M solution of cruzipain as determined by HPLC. The 27 synthetic peptides were tested in a solution inhibition assay. The K_i values were determined in the presence of the Cbz-Phe-Arg-MCA with a $k_{cat}/K_m = 6000$ for cruzipain [10]. A K_i ~ 0.1–0.2 μM was measured for the best inhibitors (Table 1). The most active inhibitors were all among those found directly by screening of the library. Peptide **1** with the sequence RIWrYWAV is the best inhibitor, $K_i = 0.11$ μM.

In the solution inhibitor studies the following was observed. The residue assumed to occupy P_4 was found not to be essential as confirmed by comparing **2** with **16** where a substitution of Cys with Arg has no effect. The Ile in P_3 was essential and could be substituted with Phe/Tyr only. In P_2 a Trp was most frequently observed but could be substituted with Phe/Tyr or even Phe(NO_2) as in **23**. The complete selectivity in P_1 for the arg over a lys residue could, in the case of cathepsin B, be explained by the formation of a salt bridge to Glu122 (Figure 4). The substitution of arg in **24** with an orn (**26**) or phe(NH_2) (**25**) residue did not enhance inhibition. In P_1' a Tyr was most frequently found but other residues were observed. Comparison of **24** and **22** shows that Phe(NH_2) is marginally better than Tyr in P_1'. The P_2' position prefers Trp, Arg or lipophilic residues and comparison of **24** and **21** show that a Phe(NH_2) could be advantageous. P_3' does not demonstrate any particular preference; however, substituting with a D-amino acid in the unimportant sites P4 and P3′ as in **24** compared with **17** and **19** reduces the inhibition significantly. In conclusion, the best inhibitors were found directly by the screening procedure.

In order to investigate whether selectivity could be obtained, the best inhibitors were assayed as inhibitors against cathepsin L, cathepsin B and kallikrein. The results are presented in Table 3. The results showed that the inhibitors were quite selective for the cysteine proteases but the selectivity differences for cathepsin B, cathepsin L and cruzipain were only 2–10 fold. However, a series of general reversible cysteine inhibitors was identified and may be used for affinity purification, for example.

The best inhibitor **1** for cruzipain was also a good inhibitor for cathepsin B and L. In order to study the mode of action of the inhibitors it was therefore attempted to fit the inhibitor **1** using constrained MD calculations and energy minimization into the active site of the crystal structure of cathepsin B [31]. It was found not to be possible to fit the substrate in the usual binding mode for all L-amino acid substrates due to steric interaction imposed by the arg residue. However, by performing the MD calculation with several different sets of weak constraints between amino acid side chains in the substrate and the pockets of the enzyme it was possible to obtain a single low-energy complex with all the side chains buried in the pockets. The

Table 2 Synthesized Analogues to the Isolated Inhibitors. Stabilization with Terminal D-Amino Acids

Comp.	Inhibitor positions corresponding to enzyme subsites S4–S4′								K_i	Found	Calc.
	P4	P3	P2	P1	P′1	P′2	P′3	P′4	(μM)	M + H	M
15	R	I	W	r	Y	W	H	V	0.22	1214.9	1214.5
16	R	I	W	r	Y	W	R	V	0.24	1234.4	1233.5
17	A	I	Y	r	Y	R	V	V	1.41	940.2	939.1
18	A	I	W	r	Y	R	A	V	1.77	1034.1	1033.3
19	R	I	W	r	Y	R	F	V	1.80	1195.6	1194.5
20	R	I	Napht	r	Y	R	F	V	6.79	1206.6	1134.4
21	a	I	W	r	Y	F-NH_2	f		6.2	1017.8	1016.2
22	a	I	W	r	F-NH_2	R	f		25.9	1009.5	1009.2
23	a	I	F-NO_2	r	Y	R	f		34.4	1016.8[a]	1016.2
24	a	I	W	r	Y	R	f		35.8	1010.7	1010.2
25	a	I	W	f-NH_2	Y	R	f		64.2	1017.3	1016.2
26	a	I	W	orn	Y	R	f		119.8	969.8	968.2
27	a	G-^{T}Bu	W	r	Y	R	f		162.6	1011.3	1010.2

[a]A peak corresponding to loss of oxygen due to photoreduction of the nitro-group was observed.

Table 3 Comparison of the Inhibitory Activity for Some of the Best Inhibitors from Tables 1 and 2 with Respect to Inhibition of Cruzipain, Cathepsin B, Cathepsin L and Human Tissue Kallikrein

		K_I (μM)			
Comp.	Sequence	Cruzipain	Cathepsin B	Cathepsin L	*h*-Kallikrein
1	RIWrYWAV	0.11	0.30	0.18	10,000
3	WIYrWFFV	0.36	0.26	0.10	10,000
4	YFFrMFGV	0.36	0.31	0.97	10,000
21	aIWrYF(NH_2)f	6.2	7.41	0.65	10,000

conformation of the enzyme backbone was maintained while contact side chains of the active side were flexible. The side chains are positioned against the bottom of the binding cleft lifting the backbone of the inhibitor 5.3 Å away from the active sulphhydryl nucleophile. The result is presented in Figures 3 and 4. The presented binding mode could explain why the inhibitors are able to bind the three enzymes almost equally well by induced fit using the flexibility of the contact side chains.

General Considerations

A conceptually new strategy, based on the solid-phase library method, was developed to identify cruzipain inhibitors. Using a single D-amino acid a library of a novel type of potential inhibitors was obtained. Using fluorescent quenched substrates as an indicator of proteolysis on the solid phase, the assay required a very limited amount of enzyme per assay. A first generation of cruzipain inhibitors was obtained, yielding compounds with K_i at the 100 nM range. Similar inhibition was observed for cathepsin B and L but not for kallikrein. Molecular modelling indicated a different binding mode for this new type of inhibitor.

EXPERIMENTAL PART

NN-Dimethylformamide (DMF) was DNA-grade from Labscan and was used without purification. Piperidine, acrylamide, *N*-ethylmorpholine (NEM) and *N*-methylimidazole were from Sigma. The Fmoc-amino acids and pentafluorophenyl esters were from Bachem (Switzerland) and NovaBiochem (Switzerland). Bis-aminopropyl ethylene glycol 1900, mesitylene-sulphon-1-yl-3-nitro-1,2,4-triazole (MSNT) and *O*-benzotriazo-1-yl-*N*,*N*,*N*,′*N*′-tetramethyl uronium tetrafluoroborate (TBTU) were from Fluka. Beads were collected from a glass plate by adherence to a closed glass capillary under an Optical Star Fluorescence Microscope with a 320 nm band pass filter for the excitation and a 410 nm low-pass filter for the detection of emitted light. Collected beads were placed directly on a sequencer cartridge filter (400379). Peptides were sequenced on an Applied Biosystems Sequencer, model 477A, equipped with an on-line phenylthiohydantoin-(PTH)-amino acid HPLC, model 120A using the program 'Micro' supplied by the manufacturer. Peptides were analysed by analytical HPLC and MALDI mass spectrometry. Enzyme assays in solution were carried out by monitoring the hydrolysis of fluorogenic peptide substrate measuring the aminomethyl coumarin (AMC) at λ_{em} 380 nm and λ_{ex} 460 nm in a Hitachi F-2000 spectrofluorimeter using a 1 ml quartz cells. The hydrolysis of the chromogenic substrate was monitored by measuring the *p*-nitroaniline (pNA) at λ 410 nm in a Hitachi U-3210 spectrophotometer. All washing procedures in the peptide synthesis were with six volumes of solvent when not otherwise stated. Fmoc cleavage was performed with 20% piperidine/DMF. Preparative reverse-phase HPLC separations were performed on a Waters HPLC system using a Delta PAK C-18 column (15 μm, 300 Å 25 × 200 mm^2) with a flow rate of 10 ml/min, or a Delta PAK C-18 column (15 μm, 300 Å, 47 × 300 mm^2) with a flow rate of 20 ml/min, and a detection at 215 nm with a photodiode array detector (Waters M 991). Solvent system A: 0.1% TFA; B: 0.1% TFA in 90% acetonitrile–10% water. Analytical HPLC was performed on a Waters system with an RCM 25 × 10 module containing a 25 NV C_{18} column was used for analysis. Buffers were as above. MALDI-TOF MS were run on a MAT-2000 from Finnigan using a matrix of α-cyano-4-hydroxycinnamic acid with bradykinin (H-1970, Bachem) or renin substrate (M-1025, Bachem) as internal references.

Synthesis of the Inhibitor Library (1)

A beaded $PEGA_{1900}$ resin was prepared as previously described [24] by inverse suspension polymerization with slow stirring (100 rpm) of a mixture of mono- and bis-acrylamido–PEG_{1900} (60 g, 0.77 equiv. acryloyl chloride added) and acryl amide (5 ml) to obtain large beads (700 μm diameter; this resin has now become available from Polymer Laboratories, UK). The loading was determined to be 0.12 mmol/g. The dried resin (1.17 g, 0.27 mmol) was packed in a syringe synthesizer. It was swelled in DMF, washed with 20% piperidine/DMF (2 volumes) and with DMF and excess solvent was removed. A solution of hydroxymethyl benzoic acid (61 mg, 1.5 equiv.) and Fmoc-Lys(Boc)-OH (63 mg, 0.5 equiv.) in DMF (11 ml) was activated for 5 min with TBTU (173 mg, 2 equiv.) and NEM (68 μl, 2 equiv.) and added to the resin. After 24 h the resin was washed with DMF and dichloromethane and was treated with 50% TFA/dichloromethane (2 volumes, 1 and 20 min). It was washed with dichloromethane and DMF and the free amino groups of the lysine side chains were reacted with Boc-Abz-ODhbt (154 mg, ~3 equiv.). The Fmoc group was cleaved and peptide synthesis was continued as previously described using Fmoc-amino acid OPfp esters (3 equiv.) with addition of a catalytic amount of Dhbt-OH (~5 mg), affording the peptide Ac-Y(NO_2)KLRFSKQK(Abz)-PEGA. The acetylation of the peptide was performed with Ac-ODhbt. The resin was washed with dichloromethane and sucked dry. It was treated with 0.1 M NaOH, washed with water and freeze dried.

Fmoc-Val-OH (570 mg, 4 equiv.) dissolved in 12 ml dichloromethane was activated with MSNT (500 mg, 4 equiv.) and *N*-methyl imidazole (102 μl, 3 equiv.) and added to the resin. After 2.7 h it was washed with dichloromethane and DMF and the Fmoc group was cleaved. Amino acid analysis of a sample showed the ratio between Val and the amino acids of the substrate to be 2 : 1. The resin was transferred to a 20 column library generator with a mixing chamber above the columns and vacuum- and pressure regulation of reagent flow. A heptapeptide library containing D-amino acids at position 4 was synthesized by standard procedures as previously described [24] using Pfp esters (3 equiv.) and Dhbt-OH catalyst, except for the D-amino acids. Fmoc-lys(Boc)-OH and Fmoc-arg(Pmc)-OH, which were activated with TBTU and NEM. In each cycle after the Fmoc cleavage using 20% piperidine in DMF and washing, the synthesizer was turned upside down and the resin was mixed by vigorous agitation on a shaking table. The resin was washed with DMF and dichloromethane and treated with three portions of 95% aqueous TFA (10 min, 10 min and 5 h). It was washed with DMF, 20% piperidine/DMF (1 volume), DMF and dichloromethane and was freeze dried. A few beads were collected and each bead was cleaved with base and the filtrate neutralized and analysed by MALDI-TOF mass spectrometry. Single peaks were detected in the mass range 700–1100.

The Enzyme Inhibitor Library Assay

The inhibitor library resin (in five portions of 0.5 g, ~250,000 large beads) was equilibrated with NaOAc 25 mM, EDTA 2 mM, pH 5.0, NaCl 0.2 M, DTT 2 mM and immediately treated with cruzipain 0.5 μM. Aliquots from the resin were inspected under a fluorescence microscope. After 5 and 24 h incubation, the reaction was terminated by filtering and washing with water, 2% aqueous TFA, water, 2% $NaHCO_3$ and water. The resin was freeze dried and aliquots (50 mg) were plated as a slurry in water on a small Petri disk for collection of beads under the fluorescence microscope. Dark beads were transported to the dry glass in the periphery of the slurry and collected with the dry end of a closed capillary. Each bead was cut into four pieces and analysed twice by Edman degradation/gas-phase sequencing and by MALDI-TOF-MS after treatment with hydrazine. The putative resin-bound inhibitors were identified and the result is presented in Table 1.

Multiple Column Peptide Synthesis of Putative Inhibitors

Peptides were synthesized on high-capacity [30] $PEGA_{1900}$ resin (0.45 mmol/g 75 mg/column) derivatized with ethylene diamine and hydroxymethyl benzoic amide. The first amino acid was attached by the MSNT procedure [27] and peptide assembly was carried out as a multiple column peptide synthesis (MCPS) [32]. A standard Fmoc amino acid Pfp ester (3 equiv.)/Dhbt-OH protocol with 20% piperidine in DMF for deprotection was used. The D-amino acid, Fmoc-arg(Pmc)-OH, was coupled as the free acid (2.5 equiv.) by *in situ* activation with TBTU [33]. The protecting groups were cleaved off while the peptides were still attached to the resin by treatment for 2 h with 95% aqueous TFA. It was filtered, washed with 95% aqueous TFA, with dichloromethane, with 20% piperidine, with DMF and with dichloromethane. The resin was dried and the peptides were cleaved

from the resin in a 2 h reaction with sodium hydroxide (0.1 M). The released peptides were filtered off and after washing the resin the solutions were neutralized to pH 7 on pH paper with HCl (0.1 M). The crude products were extracted with DMF and analysed by analytical HPLC (40 min gradient from 0 to 100% B). All the crude compounds eluted as a single major peak were purified to homogeneity by preparative HPLC (yield 20–25 mg of each). The resulting peptides had the right composition according to amino acid analyses and were further analysed by MALDI-TOF-MS.

Solution Assay of the 27 Synthetic Enzyme Inhibitors

The fluorogenic substrate Z-FR-MCA for cruzipain, cathepsin B and cathepsin L and the chromogenic substrate H-pFR-pNA for human tissue kallikrein were used to assay the peptide inhibitors. Cruzipain was assayed at 37 °C, 100 nM Na_2HPO_4/NaH_2PO_4, 10 mM EDTA, 5 mM DTT, 400 mM NaCl and 2 mM Cys, pH 6.3. Cathepsins were assayed at 37 °C in 50 mM Na_2HPO_4/NaH_2PO_4, 5 mM EDTA, 5 mM DTT, 200 mM NaCl and 2 mM Cys, pH 6.0 (cathepsin B) and 5.8 (cathepsin L). Human tissue kallikrein was assayed at 37 °C in 50 mM Tris–HCl, 1 mM EDTA, pH 9.

The substrate concentrations used in the inhibition kinetic measurement were about 10 K_m, namely, 10 μM (cruzipain), 22 μM (cathepsin L), 80 μM (human tissue kallikrein) and 900 μM (cathepsin B), respectively. The substrate hydrolysis was limited to 10% of the total substrate employed with the use of six inhibitor concentrations for each enzyme. The K_i values were calculated from $K_{i\text{-app}}$ and the substrate K_m values [34] and are presented in Table 2.

Docking of the Inhibitor 20 into the Active Site of Cathepsin B

Docking was performed using the Amber forcefield (Insight II (95)), and the crystal structure of cathepsin B [31] retrieved from the Brookhaven Protein Data Base. The inhibitor was manually aligned with the active site in an extended conformation. Different starting positions and constraint sets (12 constraints each) between ligand and enzyme were tested. The backbone and non-contact residues of the enzyme were fixed while contact residues and ligand were flexible and trial MD simulations of 5000 steps (5 ps) at 1000 K were performed for each constraint/position set. The most successful trial run placed the arg in P1 as expected and the results was used in a 100 ps (100,000) step MD-simulation at 500 K with only a fixed backbone and six inter-ligand/enzyme constraints of 4 kcal/mol each. The resulting complex was subjected to 10,000 steps of energy minimization (steepest gradient) with a fixed enzyme backbone. The results are presented in Figures 3 and 4.

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