Novel Aza Peptide Inhibitors and Active-Site Probes of Papain-Family Cysteine Proteases

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Recent characterization of multiple classes of functionalized azapeptides as effective covalent inhibitors of cysteine proteases prompted us to investigate O-acyl hydroxamates and their azapeptide analogues for use as activity-based probes (ABPs). We report here a new class of azaglycine-containing O-acylhydroxamates that form stable covalent adducts with target proteases. This allows them to be used as ABPs for papain family cysteine proteases. A second class of related analogues containing a novel O-acyl hydroxyurea warhead was found to function as covalent inhibitors of papain-like proteases. These inhibitors can be easily synthesized on solid support, which allows rapid optimization of compounds with improved selectivity and potency for a given target enzyme. We present here one such optimized inhibitor that showed selective inhibition of falcipain 1, a protease of the malaria-causing parasite, Plasmodium falciparum.

Introduction

The papain family or CA clan is one of the largest subfamilies of the cysteine proteases. Interest in this family continues to grow as a result of recent advances in our understanding of their involvement in several physiological and pathological processes.[1] Specifically, members of the lysosomal cysteine cathepsins have been shown to be involved in various human systemic diseases such as rheumatoid arthritis[2] and tumor invasion.[3] Additionally, papain-family proteases play an important role in many types of parasitic infections.[4] For example, the human malaria parasite, Plasmodium falciparum, expresses three papain-like cysteine proteases: falcipains 1, 2, and 3. The latter two primarily facilitate the degradation of hemoglobin during normal catabolism, while the function of falcipain 1 is not as clear. Falcipain 1 function has in part been defined through the use of selective inhibitors.[5]

Cysteine proteases also serve as ideal targets for development of small-molecule therapeutic agents. Cysteine-protease inhibitors have gained considerable attention over the last couple of decades and many classes of compounds are currently in human clinical trials for a number of diseases. The majority of cysteine-protease inhibitors make use of reactive functional groups that selectively modify the key active-site cysteine residue.[6,7] Examples of well-characterized inhibitors include peptidyl aldehydes and nitriles, which can react with the active-site cysteine residue to form reversible covalent adducts, as well as vinylsulfones, peptidyl epoxyxysuccinates, halomethyl ketones, and acyloxymethyl ketones (AOMKs), all of which form irreversible covalent adducts between enzyme and inhibitor.

In addition to their use for potential therapeutic applications, several classes of covalent inhibitors have been equipped with a variety of tags that allow them to be used as activity-based probes (ABPs). Such probes can specifically modify active proteases in complex proteomes thereby allowing the regulation of protease activity to be monitored with simple biochemical methods.[8] ABPs also allow affinity purification of corresponding enzymes.[9] Our laboratory and others have extensively investigated epoxysuccinates,[5,9–11] vinyl sulfones,[9,12] and, more recently, AOMKs[13] as ABPs. While these probes have proven useful for a number of biological applications, they all have limitations resulting from complex synthetic methods or scope of targets that can be analyzed. For these reasons we set out to further diversify the current cysteine-protease probe set by examining the utility of other reactive functional groups and peptide scaffolds.

We chose to focus on the O-acyl hydroxamate reactive group as it has been shown to form selective covalent bonds with the active site of cysteine proteases.[14] This class of compounds can be synthesized by using simple chemistry that is compatible with solid-phase peptide synthesis and allows extensive diversification of structural elements that access active-site residues on both sides of the key catalytic cysteine residue.

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.
Previous efforts to develop O-acyl hydroxamates \( (A, \text{Scheme 1}) \) have resulted in the development of several commercially available inhibitors of papain-family enzymes, such as catespin inhibitor III (Scheme 1).\(^{[14]} \) Mechanistic studies indicate that active group was placed at the N-terminal end of a peptide, were readily accessible by solid-phase peptide chemistry and proved to be effective inhibitors of catespin cysteine proteases. The selectivity of all compounds could be fine-tuned by adjustment of the amino acid in the P2 position. However, as a consequence of the different reactivities of the warheads and the stability of the covalent enzyme–inhibitor complexes, only a derivative of the novel warhead \( (C) \) proved to be suitable as an ABP.

### Results and Discussion

#### Synthesis

In order to validate the azapeptide O-acyl hydroxamate warhead, we initially synthesized an azapeptide analogue of the previously published O-acyl hydroxamate catespin inhibitor III (Scheme 1).\(^{[14]} \) The hydrophobic P2 residue in combination with a glycine P1 element provided selectivity for the papain subfamily of cysteine proteases. In MW4, the glycine residue was substituted by itsaza counterpart; this resulted in the novel warhead \( (B) \) (Scheme 1). In addition, the carbobenzoxy (Cbz) group was replaced by a N-acetyltyrosine that facilitated the construction of an ABP by introduction of a \(^{125}\)I radiolabel.

The synthesis of MW4 is outlined in Scheme 2. In short, properly protected dipetide methyl ester 1 was converted to carbazate 2, followed by installation of the warhead in a three-step, two-pot procedure. Isocyanate 3, formed in situ by using carbonyldiimidazole, was treated with hydroxylamine to give compound 4 in 44% yield. Acylation of the hydroxyl function with \( p \)-anisoyl chloride resulted in protected inhibitor 5. Finally, deprotection of the tyrosine side chain with 95% TFA and purification by HPLC afforded MW4 in 21% yield.

To enable rapid synthesis of MW4 and its analogues, the feasibility of (semi)solid-phase synthesis was examined. We reasoned that the oxygen of the hydroxylamine moiety would be a suitable handle for attachment to a solid support. This strategy only required the final acylation and deprotection of amino acid side chains to be executed in solution after elongation of the peptide part and cleavage from the resin.

Thus, N-Fmoc hydroxylamine was loaded onto chlorotrityl resin as described,\(^{[18]} \) deprotected with piperidine (20%), and

\[\text{MW4} \rightarrow \text{MW4}^{[125]}\]
treated with carbonyldiimidazole. It was then treated with hydrazine to furnish functionalized resin 7 (Scheme 3). Next, solid-phase peptide synthesis was performed by using DIC and HOBt as condensing agents. After capping of the amino terminus by using acetic anhydride and cleavage from the resin, which was mediated with TFA (1%), the resulting free hydroxyl function was acylated with anisoylchloride. Full side-chain deprotection was achieved with 95% TFA, after which HPLC purification yielded the desired inhibitors.

Scheme 4 lists the compounds (MW4, MW20, MW21) obtained by this method, which vary in the P2 amino-acid side chain. The same strategy could be employed to synthesize control compounds MW2 and MW22 containing the O-acyl hydroxamate warhead. Instead of carbonyldiimidazole and hydrazine, Fmoc–glycine was coupled to the resin-bound hydroxylamine, followed by elongation by using similar protocols as described.

At this point, we turned our attention to the design of an O-acyl hydroxamate-like warhead at the N terminus of a peptide. This strategy would allow on-resin acylation of the hydroxylamine functional group (i.e., formation of the warhead), which renders further synthetic steps in solution unnecessary. The

Scheme 3. Reagents and conditions: a) step 1: piperidine/DMF (1:4), 15 min; step 2: carbonyldiimidazole (6 equiv), CH2Cl2, 3 h; step 3: hydrazine (60 equiv), DMF, 1 h. b) Solid-phase peptide synthesis by using: step 1: Fmoc–amino acid (3 equiv), DIC (3 equiv), HOBt (3 equiv), 2 h; step 2: piperidine/DMF (1:4), 15 min; step 3: final capping with Ac2O (10 equiv), DIEA (10 equiv), DMF, 15 min. c) 1% TFA in DCM. d) p-Anisoyl chloride (1.9 equiv), pyridine (2 equiv), THF, 4°C, 2 h. e) TFA/TIS/H2O (95:2.5:2.5), 1 h.

Scheme 4. Overview of the synthesized inhibitors and ABPs with the different warheads A) O-acyl hydroxamate, B) O-acyl hydroxyurea, and C) azaglycine O-acylhydroxamate.
particular sequence of immobilized peptide 12 (Scheme 5) was chosen for its direct analogy to DCG-04, a previously validated ABP for papain-family cysteine proteases. Treatment of the free N terminus of the immobilized peptide with carbonyldiimidazole at room temperature gave hydantoin 13 (Scheme 5). The formation of this type of structure has previously been described in the synthesis of resin-bound azapeptides. However, hydantoin formation could be completely abolished by treatment of CDI with the free amino terminus of the resin-bound peptide at 4 °C for 5 min. To complete the formation of N-hydroxyurea 14, the resin was washed with DMF and treated overnight with hydroxylamine hydrochloride in the presence of DIEA. Finally, the free hydroxyl was acylated and probes were cleaved from the resin with TFA (95%) followed by HPLC purification (42–52% overall yield).

Evaluation of inhibitors

We initially tested representative examples of each of the warheads as inhibitors of purified cathepin cysteine proteases. Table 1 shows the kinetic inhibition constants determined by using previously described protocols. Interestingly, the aza-glycine O-acyl hydroxamate (MW4) was considerably more potent against both cathepins B and L than the original O-acyl hydroxamate (MW2). The novel O-acyl hydroxyurea (MW6bio) showed increased selectivity for cathepin L compared to the other two analogues (MW2, MW4).

To evaluate the different warheads in a more physiologically relevant setting, we performed competition-binding studies with crude homogenates of rat liver, which contain a number of characterized cathepin-protease activities. Rat-liver homogenate was incubated with increasing concentrations of MW2, MW4, and MW6. After incubation for 30 min the residual cathepin activity was determined by treatment with radiolabeled ABP DCG-04 followed by analysis of samples by SDS-

**Table 1.** Inactivation constants $K_{inact}$ [m$^{-1}$s$^{-1}$] for bovine cathepsin B and human cathepsin L.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Cathepsin B</th>
<th>Cathepsin L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW2</td>
<td>6000</td>
<td>7400</td>
</tr>
<tr>
<td>MW4</td>
<td>75000</td>
<td>65000</td>
</tr>
<tr>
<td>MW6bio</td>
<td>1400</td>
<td>43000</td>
</tr>
</tbody>
</table>

**Scheme 5.** Reagents and conditions: a) solid-phase peptide synthesis. b) step 1: piperidine/DMF (1:4), 15 min; step 2: carbonyldiimidazole (6 equiv), DMF, room temperature. c) step 1: piperidine/DMF (1:4), 15 min; step 2: carbonyldiimidazole (6 equiv), DMF, 4 °C, 5 min; step 3: hydroxylamine (10 equiv), DIEA (10 equiv), DMF, overnight. d) $p$-Anisoyl chloride (1.9 equiv), pyridine (2 equiv), THF, 4 °C, overnight. e) TFA/TIS/H$_2$O (95:2.5:2.5), 1 h.
PAGE. The resulting data were quantified by using image analy-
sis software. Concentrations at which 50% of the enzyme activi-
ty was inhibited (apparent IC50 values) are given in Table 2.

Interestingly, the azaglycine-containing inhibitor, MW4, showed nearly ten-times higher potency for all the labeled ca-
thepsins compared to the original O-acyl hydroxamate MW2. MW6, containing the novel O-acyl hydroxyurea warhead, dis-
played similar inhibitory potency as MW2.

The selectivity of MW2 for ca-
thepsin B was fivefold lower than the aza-analogue MW4. On the other hand, MW6 shows a slight preference for cate-
hepsin Z. This difference in selectivi-
ty can be explained by a combi-
nation of the nature of the war-
head and P2 position.

To confirm that these inhibi-
tors could be optimized to target specific proteases, we syn-
thetized a series of compounds with variation of the P2 posi-
tion. These compounds were screened for inhibition of falci-
pain 1, a papain-fold cysteine protease found in the human malaria parasite P. falciparum. Inhibitors were designed by using previously reported P2 residues that conferred selectivity for falcipain 1. Accordingly, MW20 was synthesized with a P2 leucine, which displays general affinity for all three falcipains.

MW21, MW22, and MW23 were synthesized with 4-methylphe-
nylalanine as the P2 element (Scheme 4). This structural ele-
ment has previously been shown to induce selectivity for falcipain 1.

Compounds (cathepsin inhibitor II, the previously reported epoxysuccinyl-based falcipain 1 inhibitor YA29, and MW20–
23) were screened by using competition-binding assays in crude cellular extracts from P. falciparum. This assay allowed quantification of potency and selectivity of each compound for the falcipain targets. Lysates were incubated with serial dilu-
tions of inhibitor for 30 min, and residual protease activity was monitored by addition of radiolabeled DCG-04 and analyzed with SDS-PAGE. Two typical sets of results are depicted in Figure 1. These data confirm that MW21 has a much higher selectivity for falcipain 1 than the leucine-containing analogue, MW20.

Quantification of the gel data through densometry allowed the calculation of the apparent IC50 values (Table 3). YA29 showed low micromolar and selective activity against falcipain 1. Commercially available cathepsin inhibitor III and azapeptide O-acyl hydroxamate MW20 displayed high nanomolar activity—a significant increase in potency compared to YA29. However, no selectivity for any of the falcipains was observed.

Installation of 4-methylphenylalanine in the P2 position, as in MW21 and MW22, restored falcipain 1 selectivity while retaining the overall increased potency relative to YA29. Although less potent, MW23 retained considerable selectivity for falcipain 1; this supports the importance of the P2 position in the design of falcipain 1 specific inhibitors.

**Table 2.** Apparent IC50 values of inhibitors for different cathepsins in rat-liver homogenates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cathepsin Z</th>
<th>Cathepsin B</th>
<th>Cathepsin H</th>
<th>Cathepsin J/C</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW2</td>
<td>1.1</td>
<td>0.47</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>cat B (2.3-fold)</td>
</tr>
<tr>
<td>MW4</td>
<td>0.17</td>
<td>0.013</td>
<td>0.45</td>
<td>0.95</td>
<td>cat B (13-fold)</td>
</tr>
<tr>
<td>MW6</td>
<td>0.13</td>
<td>0.33</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>cat Z (2.5-fold)</td>
</tr>
</tbody>
</table>

**Figure 1.** Competition experiment in lysate of P. falciparum by using decreasing concentrations of the indicated inhibitor. Radiolabeled general cysteine protease ABP DCG-04 was added to monitor remaining falcipain activity. MW21 appears to be more selective for falcipain 1 than MW20.

**Table 3.** Apparent IC50 values (μM) of inhibitors for falcipains.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Falcipain 1</th>
<th>Falcipain 2/3</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>YA29</td>
<td>2.0</td>
<td>&gt;25</td>
<td>&gt;13</td>
</tr>
<tr>
<td>Cathepsin</td>
<td>0.33</td>
<td>0.43</td>
<td>1.3</td>
</tr>
<tr>
<td>Inhibitor III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW20</td>
<td>0.094</td>
<td>0.068</td>
<td>0.7</td>
</tr>
<tr>
<td>MW21</td>
<td>0.073</td>
<td>1.6</td>
<td>22</td>
</tr>
<tr>
<td>MW22</td>
<td>0.064</td>
<td>1.0</td>
<td>16</td>
</tr>
<tr>
<td>MW23</td>
<td>1.8</td>
<td>&gt;25</td>
<td>&gt;13</td>
</tr>
</tbody>
</table>

**Covalent modification**

To gain more insight in the mechanism of inhibition of the novel warheads, we subjected four compounds (MW2, MW4, MW6, and cathepsin inhibitor III) to kinetic measurements. To determine if all compounds form stable covalent linkages with target proteases we first treated purified cathepains with a 100-fold excess of the inhibitors, removed the compounds with repeated dilution/filtration steps, and then measured resi-
dual activity with a fluorogenic substrate. For both cathep-
sin B and L, no recovery of enzymatic activity was observed (Figure 2). This result clearly indicates an irreversible mode of inhibition, which could be a result of covalent attachment to the active site. To further corroborate this, cathepsin L was
incubated with an excess of MW2, MW4, or MW6bio and analyzed by MALDI-TOF. The spectra revealed that the molecular ion of the treated enzyme had shifted to higher molecular weight compared to an untreated sample (see Supporting Information). Unfortunately, lack of resolution and accuracy prohibited the identification of the exact nature of the adduct. However, in line with previous reports, it is likely that the peptide part of the inhibitor was covalently attached to the enzyme through a S–N bond.

Activity-based probes

The covalent nature of warheads A–C prompted us to investigate their utility as ABPs. To test the ability of the compounds to directly label cathepsins, the tyrosine residues of MW2, MW4, and MW6 were subjected to radio-iodination as described previously. Surprisingly, incubation of purified cathepsin B or rat-liver homogenates with the resulting radiolabeled probes did not show any labeling of cathepsin proteases (data not shown). This lack of labeling by radiolabeled probes was found to be the result of decomposition of inhibitors under iodination conditions, as observed by LC-MS analysis of inhibitors modified with a stable iodine isotope.

Alternatively, ABPs were obtained by synthesizing the biotinylated derivatives MW2bio, MW4bio, and MW6bio (Scheme 4). In the first two compounds, an extra aminohexanoic acid spacer and an (ε-biotinyl)lysine were incorporated at the N-terminal end of the corresponding inhibitor. Labeling of cathepsin B (Figure 3 A) was performed by incubation with the three biotinylated probes (10 μM) and the control DCG-04 probe (0.1 μM) at pH 5.5. Before loading onto a SDS-PAGE gel, samples were exposed either to reducing (pH 6.8, 150 mM DTT, 100 °C, 2 min) or nonreducing (pH 6.8, RT) conditions. As expected from its modest activity against cathepsin B in competition and kinetic experiments, MW2 proved to be a weak probe (lanes 1 and 5). MW4, which displayed higher activity in previous experiments, showed strong labeling of cathepsin B (lanes 2 and 6). Interestingly, the covalent modification formed between MW6 and the active-site cysteine was not stable when it was boiled in the presence of DTT (lane 3 vs. 7). Possibly, the "mercapto–urea" protease–ABP adduct was cleaved under reductive conditions. The lack of cathepsin B labeling by MW6 was also observed when low concentrations of DTT were included in the reaction buffer; this illustrates the high instability of the modification (data not shown).

Cathepsin L (Figure 3B) showed similar results as cathepsin B. Sensitivity of the active-site cysteine to oxidation required the use of a DTT containing reaction buffer during all experiments. As a result, no labeling with MW6 was observed (lanes 3 and 7). The warhead of MW6 is therefore less suitable for general use in ABPs. On the other hand, MW4 shows effective labeling of both cathepsins and represents a novel warhead for use in activity-based proteomics.
Conclusions

In this paper, we present two novel cysteine-protease inhibitor scaffolds based on the O-acyl hydroxamate reactive group. The first comprises an azaglycine O-acyl hydroxamate, for which a semisolid-phase synthesis was devised. These compounds are efficient inhibitors of papain-family proteases and form stable covalent adducts with their targets.

Selectivity for other cysteine-protease families might be furnished by introduction of a P1 element. For example, a P1 aspartate could furnish reactivity against caspasases, which are members of the CD clan of cysteine proteases. These compounds may be accessed by solid-phase synthesis by using a recent procedure in which azapeptide probes are attached to the resin through their P1 side-chain linkage.20

The second type of inhibitors, comprising the O-acyl hydroxurea warhead, could be fully synthesized on resin, thus presenting a significant advantage over the other inhibitors. Although mass spectrometry data support the covalent nature of inhibition for all warheads, the complex resulting from O-acyl hydroxamates, however, proved to be suitable for labeling different cathepsin protease activities and therefore represents a novel class of ABPs.

Experimental Section

General methods: Unless otherwise noted all resins and reagents were purchased from commercial suppliers and used without further purification. All solvents used were of HPLC grade. Reversed-phase HPLC was conducted on a C18 column by using an AKTA explorer 100 (Amersham Pharmacia Biotech). LC-MS data were acquired by using an API 150EX system (Applied Biosystems).

Synthesis: See Supporting Information for detailed description of the synthetic procedures.

Competition in whole proteomes: Rat-liver homogenates were used (1 mg/mL) in an acetate buffer (50 mM, pH 5.5). Samples were incubated with the indicated concentrations of inhibitor at room temperature for 0.5 h and subsequently treated with radiolabeled DCG-04 (100 cpm) for an additional 0.5 h prior to subject to gel-electrophoresis (12% polyacrylamide). Visualization of protease activities was performed by using a Typhoon Scanner for phosphorimaging. Data were quantified by using NIH ImageJ and analyzed with GraphPad Prism.

For competition in P. falciparum, synchronized cultures of the parasite were harvested at the trophozoite stage for optimal labeling of all three falcipains after saponin lysis of host red-blood cells. Parasite pellets were lyzed by addition of NP40 (1%) at pH 5.5. The insoluble fraction (40 μg) was used in each competition reaction, which was incubated with inhibitor dilutions for 0.5 h and then treated with radiolabeled DCG-04 for 1 h. Visualization and quantitation were performed as described above.

Kinetic experiments: Inhibition kinetics of cathepsins B and L were determined under pseudo first-order conditions with at least tenfold molar excess of inhibitors as described previously.19,20 Concentration of cathepsins in the assay was 1 nM (cathepsin L) and 10 nM (cathepsin B). Cathepsin activity was measured in the presence of fluorogenic substrate Z-RR-AMC (10 μM; Bachem, Torrance, CA) for cathepsin B and Z-FR-AMC (10 μM; Bachem) for cathepsin L. The concentration of DMSO during the measurement never exceeded 2.5%. The increase of fluorescence (370 nm excitation, 460 nm emission) was continuously monitored with a SpectraMax M5 fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

For irreversibility experiments, cathepsin L or B (20 nM) were incubated with inhibitor (2 μM) for 2 h at room temperature. Subsequently, the inhibitor was diluted by repeated washing/filtration steps over a Microcon YM-10 centrifugal filter (Millipore), so that free inhibitor concentration was less than 4 nM. Next, the activity of enzymes was measured as described.19

Mass spectrometry: Purified cathepsin L (approximately 5 μM) was incubated with the indicated inhibitors (20 μM; diluted from a 1000x stock in DMSO) or with control DMSO at pH 5.5 (5 mM sodium acetate) for 1 h. MALDI-TOF analysis was performed on a Bruker Ultraflex MALDI-TOF/TOF spectrometer of the Stanford Proteomics & Integrative Research Facility.

Biotin blots: Purified bovine spleen cathepsin B (100 ng; Calbiochem) or recombinant human cathepsin L (a kind gift from the Turk laboratory, Ljubljana, Slovenia) were incubated at room temperature with indicated concentrations of biotinylated ABP for 30 min (50 mM acetate buffer, pH 5.5, 1% DMSO, 5 mM MgCl2, with or without 2 mM DTT). After addition of either reducing (150 mM DTT) or nonreducing sample buffer, mixtures were run on a polyacrylamide gel and transferred to a nitrocellulose membrane. Biotinylated proteins were detected by using VectaStain reagents (Vector Labs, Burlingame, CA) and visualized on film by chemoluminescence.

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