ACS Medicinal Chemistry Letters

Letter

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ACS Med. Chem. Lett., **Just Accepted Manuscript** • DOI: 10.1021/acsmedchemlett.5b00474 • Publication Date (Web): 28 Dec 2015

ACS Publications

ACS Medicinal Chemistry Letters is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Cathepsin B Inhibitors: Combining Dipeptide Nitriles with an Occluding Loop Recognition Element by Click Chemistry

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KEYWORDS: Copper-catalyzed azide-alkyne cycloaddition, cysteine proteases, human cathepsin B, nitrile inhibitors

ABSTRACT: An active site mapping of human cathepsin B with dipeptide nitrile inhibitors was performed for a combinatorial approach by introducing several points of diversity and stepwise optimizing the inhibitor structure. To address the occluding loop of cathepsin B by a carboxylate moiety, click chemistry to generate linker-connected molecules was applied. Inhibitor **17** exhibited K_i values of 41.3 nM, 27.3 nM or 19.2 nM, depending on the substrate and pH of the assay. Kinetic data were discussed with respect to the conformational selection and induced fit models.

Cathepsin B is a papain-like cysteine protease that is ubiquitously expressed and involved in various physiological processes.[1] In non-tumor cells, it is localized within endosomes/lysosomes, while in transformed cells it adopts a peripheral distribution in the cytoplasm and is, moreover, associated with the plasma membrane.[2,3] At the surface of invasive tumor cells, cathepsin B was found to bind to annexin II or caveolin-1, the structural protein of caveolae. Caveolae serve as a platform for a proteolytic cascade for the degradation of the extracellular matrix (ECM), executed by several proteases including cathepsin B.[2-4]

Cathepsin B affects the ECM either directly by extracellular proteolytic degradation of its components or indirectly via activation of others proteases. Partially degraded ECM components can be internalized to intracellular endosomal/lysosomal versicles where the proteolysis is continued. This ECM breakdown remodels the tumor environment, promotes tumor invasion, and enables angiogenesis and metastasis. Cathepsin B also contributes to angiogenesis by degrading metalloprotease inhibitors and releasing growth factors that are bound to ECM components.[2,5,6] The crucial roles of cathepsin B at multiple points of the tumor development have been established in several *in vitro* and *in vivo* models and cathepsin B was proposed to be a prognostic marker in patients with various types of cancer.[1,2,7]

Cathepsin B shows endopeptidase and exopeptidase activity, a unique feature for cysteine cathepsins, arousing from a flexible structural element, referred to as the occluding loop. The occluding loop is a 19-20 amino acid section that blocks the active site cleft at the end of the primed site. This event leaves only space for two amino acid residues of the substrate C-terminal of the scissile bond, *i.e.* in P1' and P2' position. In this so called closed conformation, two salt bridges, His110-Asp22 and Arg116-Asp224, hold the occluding loop over the primed subsites of the substrate binding cleft, preventing extended binding of large endopeptidase substrates and conferring an exopeptidase activity to cathepsin B. Mutations of His110 and Asp22 or the deletion of twelve amino acids of the occluding loop to enforce an open conformation reduced the exopeptidase activity of cathepsin B in favor of its endopeptidase activity.[8,9] His110 and His111 of the occluding loop contribute to the exopeptidase activity by providing an appropriately spaced acceptor to bind the C-terminal carboxyl group of a peptide substrate.[2,3,10,11]

Low pH values of around 4-5 are connected with the exopeptidase activity of cathepsin B, while the endopeptidase activity increases with a rising pH value. This effect was suggested to result from the protonation/deprotonation state of the residues involved in the stabilizing salt bridges. At low pH, corresponding to the conditions of lysosomal acidic compartments, the closed conformation is preferred and cathepsin B displays a carboxydipeptidase activity.[2,3,11] Beyond the localization in the lysosomes and late endosomes, the endopeptidase activity of cathepsin B is thought to predominate at pH values of 6-7.4 and to be typical for the degradation of proteins of the ECM.[2,10,11]

The introduction of an electrophilic warhead in the position of the scissile peptide bond has been a successful strategy to design peptidomimetic inhibitors. Peptide nitriles form reversible thioimidate adducts resulting from the nucleophilic attack of the active-site cysteine. As a matter of fact, such peptide nitriles can only utilize the non-covalent interactions of the P4-P1 residues with the non-primed subsites S4-S1. However, as it has been demonstrated with nitrile inhibitors for cathepsin B, a spacer can direct an acidic moiety to the S' region to allow for an advantageous salt

bridging with the histidine residues of the occluding loop.[12] Peptide nitriles attracted much attention as synthetic inhibitors for cysteine cathepsins,[13-15] in particular for the treatment of osteoporosis, where the cathepsin K inhibitor odanacatib (**I**, Figure S1 in the Supporting Information) is currently being investigated in clinical phase III.[2,3] Further warheads for reversible inhibition of cysteine cathepsins are *e.g.* peptide aldehydes or ketones, while irreversible inhibition can be achieved with *e.g.* epoxide derivatives, vinyl sulfones or acyloxymethyl ketones.[2,3,13,14,16,17] Other cathepsin B inhibitor types include nitroxoline derivatives,[6,18] redox-reactive compounds,[19] 1,2,4-thiadiazoles,[20] aziridinyl peptides,[21] and cystatin-derived azapeptides.[22]

To enhance the selectivity of the broad spectrum cathepsin inhibitor E-64 (**II**, Figure S1),[23] further epoxysuccinyl derivatives, *e.g.* the highly potent and cathepsin B-selective CA-074 and CA-030 (**III** and **IV**, Figure S1) have been developed, binding exclusively to the S1' and S2' pockets and exploiting interactions with the positively charged histidine residues of the occluding loop.[24-27] Noteworthy, such epoxide dipeptides with a free C-terminal proline exhibited stronger cathepsin B inactivation at lower pH values than under neutral conditions.[25,28]

Herein, we have used the dipeptide nitrile chemotype to map the non-primed binding region of human cathepsin B. The outcome of these experiments was utilized for a fragment-based approach by combining the appropriate structure with a preselected linker moiety to address the occluding loop of cathepsin B with a terminal carboxylic group. The so designed inhibitors were assembled via click chemistry and investigated with respect to the target's exo- and endopeptidase activity.

A library of 57 dipeptide nitriles bearing different amino acids in P2, mostly aminoacetonitrile in P1 and different capping groups in P3 position was evaluated at human cathepsin B by performing a photometric assay using pH 6.0 and Cbz-Arg-Arg-pNA as substrate. As for the inhibition of human cathepsins L, S, K, F by these compounds,[29-32] the linear progress curves revealed a 'fast-binding' behavior. The obtained IC_{50} values were converted into the K_i values using the Cheng-Prusoff equation. The kinetic results are depicted in Figure S2 and Table S1 in the Supporting Information; the K_i values span from >500 μM to 0.33 μM.

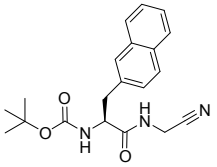
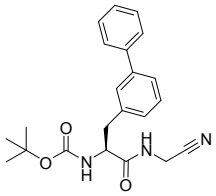
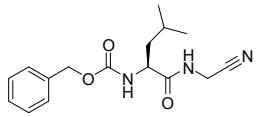
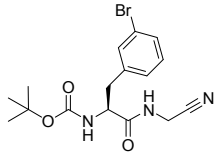
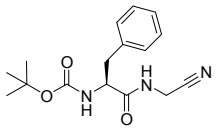
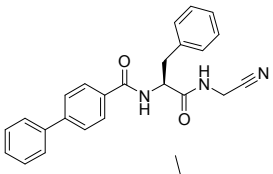
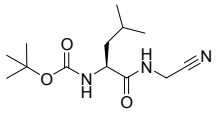
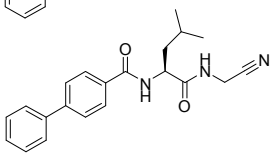
From this set of data, first structure-activity relationships were drawn. Regarding the P2 position, several compounds with a Boc capping group at P3 and glycine nitrile at P1 position as common features were compared. Five of these inhibitors, **1**-**5**, are listed in Table 1. Unnatural amino acid residues as 2-naphthylmethyl, *meta*-bromobenzyl or *meta*-phenylbenzyl caused a pronounced cathepsin B inhibition (**1**-**3** *versus* **4** and **5**). In comparison with the *meta*-connected derivatives **2** and **3**, a remarkable decrease in potency was observed for the corresponding *para*-connected isomers **68** and **70** (see Table S1 for K_i values). Furthermore, in P3 position, a more voluminous residue than the Boc capping group turned out to be advantageous for increasing the inhibitory potency. This can be exemplarily seen by comparing **5** and **6** (Table 1) where the Cbz capping group caused a 3-fold lower K_i value. A similar effect was observed when replacing the Boc group in **4** by Cbz in Cbz-phenylalanylglycine nitrile (**53**, Table S1). However, compounds with a *para*-phenylbenzoyl group in P3 position possessed a stronger inhibitory capability towards cathepsin B (**4** *versus* **7** and **6** *versus* **8**, Table 1). Compound **99** (Table S1) which shares the structure of **8** but bears a methionine side chain in P1 position was even more potent than **8** and showed the strongest cathepsin B inhibition of all 57 screened compounds (K_i = 0.33 ± 0.03 μM). This result might indicate that the introduction of a side chain in P1 position does not diminish the activity, an issue that supports our design of compounds with a linker reaching into the primed binding region of cathepsin B.

The screening results were used for the synthesis of three new inhibitors with beneficial structural features, *i.e.* a combination of the *para*-phenylbenzoyl group at P3 with either a 2-naphthylmethyl, *meta*-bromobenzyl or *meta*-phenylbenzyl moiety at P2 position. The target compounds **9**-**11** were synthesized as depicted in Scheme S1 in the Supporting Information. Dipeptides **1** and **2** (for structures see Table 1) were deprotected with methanesulfonic acid and subsequently coupled with biphenylcarboxylic acid leading to **9** and **10**. Compound **10** was further transformed in a Suzuki reaction with phenylboronic acid to generate compound **11** (for structures **9**-**11** and kinetic data with human cathepsins B, S, L and K see Table 2). Derivatives **9** and **10** were found to be potent cathepsin B inhibitors with K_i values of less than 130 nM. By replacing the *tert*-butyloxy with the biphenyl-4-yl residue, a 27- or 43-fold increase in potency was achieved (**1** and **2**, Table 1 *versus* **9** and **10**, Table 2). Compound **11** did not show the expected increase in potency toward cathepsin B (**11**, Table 2 *versus* **3**, Table 1), however, it turned out to be a strong and selective inhibitor of cathepsin K. Compounds **9** and **10** were dual cathepsin B/S inhibitors with selectivity over cathepsins L and K.

So far, we optimized the P2 and P3 substructures of the envisaged cathepsin B inhibitor. In the next step, the unique structural feature of cathepsin B, the occluding loop, should additionally be taken into consideration. The occluding loop when inserted into

Table 1. Inhibition of Human Cathepsin B by Dipeptide Nitriles 1-8

Compound	K_i (μM)[a]	Compound	K_i (μM)[a]	Compound	K_i (μM)[a]

1		3.46 ± 0.19	**3**		5.35 ± 0.21	**6**		32
2		5.17 ± 0.37	**4**		37	**7**		3.92 ± 0.97
			5		94	**8**		1.79 ± 0.14[b]

[a] Experiments were performed at pH 6.0 with Cbz-Arg-Arg-pNA. Values without standard errors refer to duplicate experiments with a single inhibitor concentration of 50 μM. IC_{50} values were calculated by using the equation $IC_{50} = [I]/(v_0/v - 1)$, where v and v_0 are the rates in the presence and absence of the inhibitor, respectively, and [I] is the inhibitor concentration. Values with standard errors refer to duplicate measurements with five different inhibitor concentrations. IC_{50} values were determined by nonlinear regression using equation $v = v_0/(1+[I]/IC_{50})$. Standard error of the mean (SEM) values refer to this nonlinear regression. K_i values ± SEM were calculated from IC_{50} values by applying the equation $K_i = IC_{50}/(1+[S]/K_m)$, where [S] is the substrate concentration. [b] Value was taken from ref 32.

the active site cleft leads to a relatively blocked S' region and dictates the carboxydipeptidase activity of the protease. The two histidine residues can interact with a carboxylate moiety of inhibitors as has been exemplified *e.g.* with CA-074, CA-030 and peptidomimetic compounds containing a linker-connected benzoic acid moiety.[12,20,24-28] These results, together with the aforementioned observation on the potency of the P1 methionine inhibitor **99** (Table S1) justifies our next step in the course of the structural optimization. Model dipeptide nitriles were designed with a Cbz capping group, phenylalanine in P2 position and various linkers to address the carboxylate recognition site. For this purpose, a click-chemistry approach was followed using a Cu^{II} salt and sodium ascorbate as reducing agent.[33] The preparation of the model compounds **12** and **13** is illustrated in Scheme S2 (for structures see Table 2). Boc-protected serine was *O*-alkylated to introduce an alkyne moiety. It was converted to the primary carboxamide, deprotected, coupled with Cbz-phenylalanine and transformed to the nitrile. Azidoacetic acid and 3-azidopropanoic acid were prepared from appropriate bromocarboxylic acids and used for the click reaction to form the final products **12** and **13**.

Two further model compounds were designed in which the central part of the linker, OCH_2-triazole, was shifted towards the carboxylate recognition site. Essentially the same approach as to **12** and **13** was followed starting from Boc-homoserine (Scheme S3, Supporting Information) to receive compounds **14** and **15** with an extended linker length (for structures see Table 2). With the four new compounds (**12-15**) in hand, the biochemical evaluation was carried out to ascertain the optimal linker structure for the interaction of the carboxylate with the occluding loop of cathepsin B (Table 2). Although **12-15** were poor inhibitors of human cathepsin B, compounds **13-15** showed a particular weak inhibition and thus the structure of **12** was considered for our further design. The high K_i values from 24 to 196 μM determined for cathepsin B inhibition by **12-15** were highly unexpected, the more so as the other cathepsins were inhibited to a reasonable extent. Presumably, the not optimized amino acid residues in P2 and P3 position prevented a pronounced cathepsin B inhibition by **12-15**. Nonetheless, by maintaining the linker present in **12** and combining the results of our fragment-based approach, a potent cathepsin B inhibitor was finally obtained.

The synthesis of the optimized cathepsin B inhibitor **17** and the corresponding methyl ester **16** (for structures see Table 2) is shown in Scheme S4 in the Supporting Information. Furthermore, two analogs of **17** with the same P3 and P2 moieties were designed in which the length of the linker between the P1 α-carbon and the carboxylate was reduced. The corresponding final compounds **18** and **19** are listed in Table 2. Their synthesis was performed starting from the clickable amino acid Boc-proparylglycine (Scheme S5, Supporting Information).

The dipeptide nitrile **16** exhibited a general and unexpectedly strong inhibition profile towards the four investigated human cathepsins (Table 2). The extended neutral P1 residue of **16** was tolerated by the cathepsins and even provided additional affinity for cathepsins B, L and K, as can be seen from the data of inhibitor **10** and those of **16**. Next, we compared the ester **16** with the free acid **17**. The latter was 2-, 15- and 5-fold less active at cathepsin S, L and K, respectively. Similar K_i values were determined for **16** and **17** at cathepsin B. Thus, we concluded that additional benefit was not gained by the free carboxylate function and that cathepsin B is mainly present in the open conformation under the assay conditions used. Unexpectedly, **18** and in particular **19**, although optimized with respect to their P3 and P2 moieties, were only weak inhibitors when examined at pH 6.0.

It has been reported that a pH value of 6.0 and the use of 'small' substrates such as Cbz-Arg-Arg-pNA (or Cbz-Arg-Arg-AMC) caused a preferred endopeptidase activity which is exerted by cathepsin B in the open conformation.[6,8,10,18] However, as the occluding loop is considered to be a highly flexible segment, the closed conformation might be formed effortlessly and might be stabilized by inhibitors that can favorably interact *e.g.* with the histidine residues of the

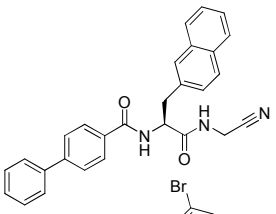
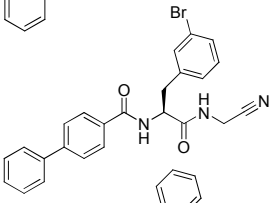
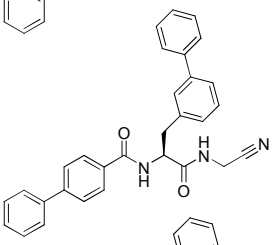
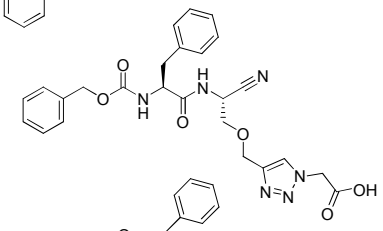
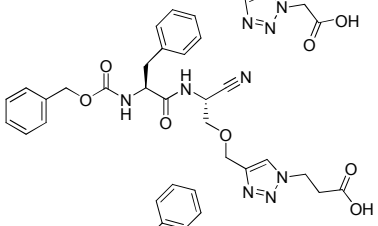
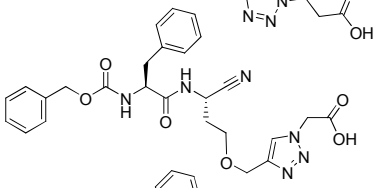
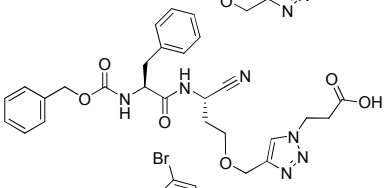
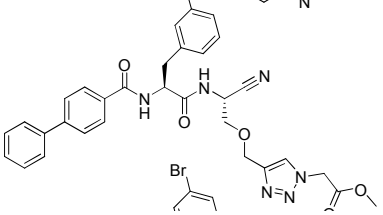
occluding loop.[12,24-27] Moreover, the pNA (or AMC) substrate only occupies a small part of the primed substrate binding region and is therefore not expected to prevent the formation of the closed conformation. Accordingly, Cbz-Arg-Arg-AMC or Cbz-Phe-Arg-AMC were also mentioned not to be appropriate model substrates to exclusively study the endoproteolysis of cathepsin B.[8,10] Mutations in cathepsin B which influence the formation of the closed conformation affected the cleavage of true endopeptidase substrates, *e.g.* Abz-Ala-Phe-Arg-Ser-Ala-Ala-Gln-EDDnp, cleaved after arginine, but had little effect on the hydrolysis of Cbz-Phe-Arg-AMC.[8] Moreover, peptidic inhibitors with a linker-connected benzoic acid moiety targeting the occluding loop have been reported to be highly active under assays conditions (pH 5.8, Cbz-Arg-Arg-AMC)[12] similar to those that we have used (pH 6.0, Cbz-Arg-Arg-pNA).

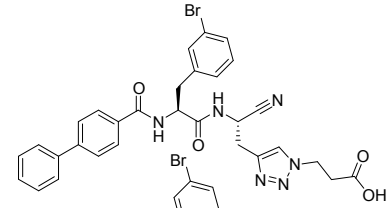
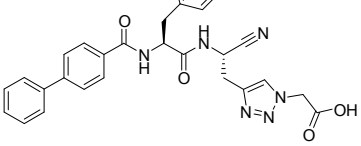
In order to address the effect of pH on the inhibitory activity of our compounds **17-19**, we repeated the measurements at pH 4.5. The kinetic data of cathepsin B inhibition by **17-19** and, for reasons of comparison, **10** and **16** are given in Table 2. When performing the assay at pH 4.5 and maintaining the 'small' substrate Cbz-Arg-Arg-pNA, a weak reduction of potency was observed for **10**, whereas the inhibition of **16** was attenuated to a stronger extent (26.0 nM at pH 6 *versus* 136 nM at pH 4.5). On the contrary, inhibitors **17-19** exhibited lower K_i values under acidic assay conditions (e.g. **17**: 41.3 nM at pH 6 versus 27.3 nM at pH 4.5). These data support the interaction of the carboxylate moiety of **17-19** with the protonated histidine residues of the occluding loop. Next, we examined the inhibition profile of the five peptide nitriles when an exopeptidase substrate of cathepsin B was used. These measurements were performed with Abz-Gly-Ile-Val-Arg-Ala-Lys(Dnp)-OH at pH 4.5. The 'quenched' fluorescent substrate is cleaved after arginine and provides the free terminal carboxylate moiety for salt bonds to the occluding loop. Whereas **10** and **16** exhibited a similar K_i value as before, the inhibition of cathepsin B by **17-19** was further enhanced (e.g. **17**: K_i = 19.2 nM). Nevertheless, the unfavorable reduction of the linker length could not be overcome, since **18** (K_i = 132 nM) was 7-fold and **19** (K_i = 368 nM) was 19-fold less active than **17**.

Our data indicate that the acids, for example **17**, but not the corresponding ester **16**, can gain affinity when the equilibrium of cathepsin B is shifted to the closed conformation, where the exopeptidase character predominates. The enhanced affinity is assumed to result from the favorable ionic contacts between the carboxylate of **17** and the occluding loop's histidines. Accordingly, the ester **16**, unable to exert such ionic contacts, lost affinity when shifting the assay conditions to pH 4.5.

The similar K_i values obtained at pH 4.5 for **10**, **16** and **17** either with the 'small' or the exopeptidase substrate led to the conclusion that the choice of the substrate did not strongly affect the equilibrium between the open and closed form of cathepsin B. Under acidic conditions, inhibitor **17** is expected to bind to this closed conformation as defined by the conformational selection model.[34] However, a still pronounced inhibition by **17** (but not by **18** or **19**) was obtained at pH 6 when the salt bridges that bind the loop to the enzyme body are thought to be disrupted and the equilibrium to be shifted to the open conformation. Under these conditions, an induced fit model might be applicable. Hence, inhibitor **17** binds to the open form of cathepsin B and causes a conformational readjustment.[34] To reach an optimal complementarity of the enzyme's binding sites and the ligand's substructures, the occluding loop adopts the closed conformation, thus providing additional ionic contacts to the inhibitor.

Table 2. Inhibition of Human Cathepsins by Dipeptide Nitriles 9-19

Compound		K_i (μM)[a]			
		Human Cathepsin B	Human Cathepsin S	Human Cathepsin L	Human Cathepsin K
9		0.126 ± 0.008[b]	0.180 ± 0.015	4.30	4.50
10		0.120 ± 0.003[b] 0.205 ± 0.022[c] 0.220 ± 0.021[d]	0.178 ± 0.009	0.609 ± 0.057	0. 930
11		1.23 ± 0.21[b]	2.66 ± 0.41	0.707 ± 0.098	0.0245 ± 0.0083
12		24.0[b]	4.48 ± 0.35	1.05 ± 0.06	0.310 ± 0.020
13		33.0[b]	2.25 ± 0.18	1.27 ± 0.06 nM	0.370 ± 0.040
14		101[b]	1.00 ± 0.16	2.16	0.580
15		196[b]	0.490 ± 0.080	1.92	0.370 ± 0.040
16		0.0260 ± 0.0029[b] 0.136 ± 0.014[c] 0.131 ± 0.010[d]	0.186 ± 0.028	0.431 ± 0.069	0.0676 ± 0.0093

17		0.0413 ± 0.0043[b] 0.0273 ± 0.0040[c] 0.0192 ± 0.0025[d]	0.352 ± 0.038	6.30	0.335 ± 0.041
18		3.64 ± 0.34[b] 0.420 ± 0.075[c] 0.132 ± 0.008[d]	1.80 ± 0.21	6.98	0.226 ± 0.049
19		11.2 ± 0.8[b] 0.707 ± 0.045[c] 0.368 ± 0.084[d]	4.23 ± 0.33	11.7	0.535 ± 0.026

[a] See Table 1 for details. Values without standard errors refer to duplicate experiments with [I] = 20 μM. [b] Assay at pH 6.0 with Cbz-Arg-Arg-pNA. [c] Assay at pH 4.5 with Cbz-Arg-Arg-pNA. [d] Assay at pH 4.5 with Abz-Gly-Ile-Val-Arg-Ala-Lys(Dnp)-OH.

It was a striking feature of our kinetic data, that the optimization of the inhibitor structure with respect to the P3 and P2 moieties made a major impact on activity. The interaction of these moieties with their corresponding subsites, S3 and S2, is thought to be the primary binding event and can occur irrespective of the open or closed conformation of the enzyme. However, an optimized P3-P2 structure could not compensate the inappropriate linker structure as present in **18** and particularly in **19**. Of course, the crucial step to elongate the residence time of the inhibitor-enzyme complex is the formation of the covalent thioimidate through the attack of the active site cysteine at the nitrile warhead. Noteworthy, when using the 'small' substrate Cbz-Arg-Arg-pNA in the assay, it can possibly bind to cathepsin B in both the closed and open conformation with similar affinity, as it could also be deduced from similar K_m values

In conclusion, we have employed a library of 57 dipeptide nitriles for a mapping of the active site of human cathepsin B. A *para*-phenylbenzoyl group in P3 and 3-bromophenylalanyl in P2 position as favorable moieties were considered to be combined in a fragment-based approach with an optimized linker structure connecting the α-carbon of the P1 position with a free carboxylate group. This structure was successfully assembled through click chemistry leading to the selective and potent covalent cathepsin B inhibitor **17**. Despite the assumption that **17** addressed the recognition site of the occluding loop, potency differences at varying assay conditions and thus at a changed equilibrium between the open and closed conformation of the target were rather small. The complex interplay of the target, substrate and inhibitor in the light of the conformational selection and induced fit models represents a subject for future investigations.

ABBREVIATIONS

Abz, 2-aminobenzoyl; AMC, 7-amido-4-methylcoumarin; Dnp, 2,4-dinitrophenyl; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine.

ASSOCIATED CONTENT

Supporting Information

Table S1, Figures S1 and S2, Schemes S1-S5. Synthetic procedures and characterization of all compounds. Enzyme inhibition assays. ^{1}H NMR and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors, who have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

J.S. was supported by the Gender Equality Center of the Bonn-Rhein-Sieg University of Applied Sciences.

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Cathepsin B Inhibitors: Combining Dipeptide Nitriles with an Occluding Loop Recognition Element by Click Chemistry

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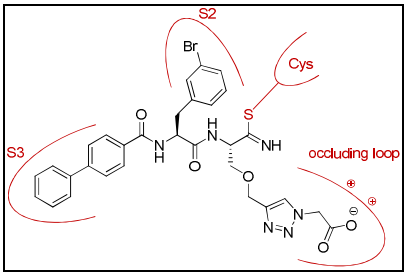

The therapeutically relevant cysteine protease cathepsin B can act both as exo- and endopeptidase, owing to the flexibility of the occluding loop and the protonation of its histidine residues. A library of dipeptide nitriles was evaluated for cathepsin B inhibition and optimized candidates were assembled in a fragment-based approach. They bear a carboxylate moiety as occluding loop recognition element.