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3,4-Disubstituted Azetidinones as Selective Inhibitors of the Cysteine Protease Cathepsin K. Exploring P2 Elements for Selectivity

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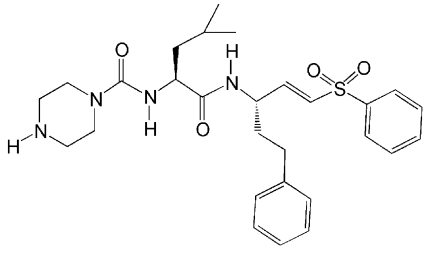
Abstract—A novel series of 3,4-disubstituted azetidinones based inhibitors of the cysteine protease cathepsin K (Cat K) has been identified. Although not optimized, some of these compounds show at least 100-fold selectivity against other cathepsins. The use of cyclic moieties as P2 elements has proven to be crucial to achieve a high degree of selectivity.

Osteoporosis, the most common form of metabolic bone disease, is a consequence of an imbalance between bone resorption and bone formation.[1] Bone resorption is carried out by multinuclear cells known as osteoclasts while osteoblasts are responsible for bone formation. Cathepsin K (Cat K) is a cysteine protease of the papain super family that is highly expressed in osteoclasts and is believed to play a key role in the degradation of the organic matrix of bone.[2] Consequently, there has been significant interest in the development of drugs to control bone loss by limiting the proteolytic activity of Cat K.

As part of our studies on the design and synthesis of Cat K inhibitors we recently reported the X-ray crystal structure of human Cat K complexed with APC-3328 (CRA-03328), a potent irreversible inhibitor of this enzyme.[3] APC-3328 (CRA-03328) is a vinyl sulphone which was found to bind to the active site in a manner that mimics substrate binding from P1 to P3 (Fig. 1).



APC-3328/CRA-03328

Figure 1.

Having succeeded in finding a highly active irreversible inhibitor of human Cat K, we focused our attention on the study of new moieties as suitable warheads to develop reversible and selective Cat K inhibitors. Certain 3,4-disubstituted azetidin-2-ones are known to bind to the active site of bacterial serine β-lactamases and D-D-transpeptidases leading to β-lactamase and bacterial cell wall synthesis inhibition, respectively.[4] Recently, 3,4-disubstituted azetidin-2-one derivatives have been reported to have excellent inhibitory properties of Cat B, L and S.[5] Based on this fact, we decided to investigate the usefulness of this warhead in the development of novel Cat K inhibitors.

Knowing that leucine is a P2 residue preferred by Cat K,[6] our first approach to achieve selectivity was to further investigate the structural differences of the S2 binding pocket among the different cathepsins by synthesizing non-natural P2 containing inhibitors. Therefore, here, we describe the synthesis and structure–activity relationship (SAR) of a series of 3,4-disubstituted azetidin-2-ones having different unnatural amino acid as P2 elements.

The designed inhibitors were synthesized as outlined in Scheme 1. Intermediate **1** was obtained from the

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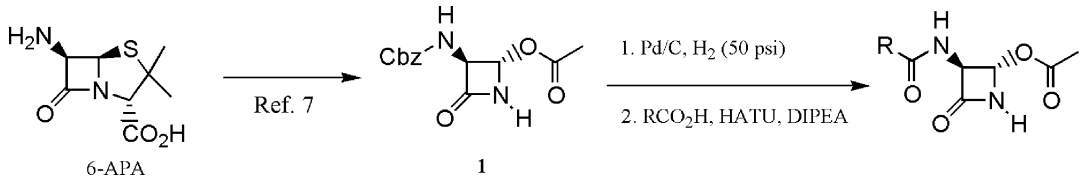


6-APA 1

Scheme 1.

Table 1.

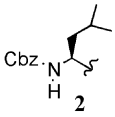
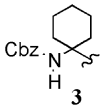
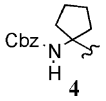
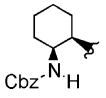
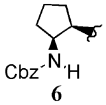
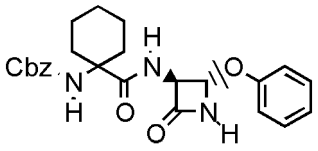
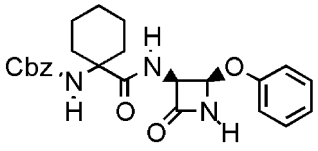
R	Cat K (μM) K_i	Cat B (μM) K_i	Cat L (μM) K_i	Cat S (μM) K_i	B/K	L/K	S/K
 **2**	0.0022	2.1	0.088	0.0092	950	40	4
 **3**	0.0057	4.3	6.8	0.95	750	1,190	160
 **4**	0.16	7.7	140	28	48	870	175
 **5**	0.0088	1.6	3.7	0.13	180	420	15
 **6**	2.1	52	>150	11	24	>70	5
 **7**	0.2	65	>150	20	320	>750	100
 **8**	0.95	120	>150	>150	120	>160	>160

Table 2.

Compd	Cat K (μM) K_i	Cat B (μM) K_i	Cat L (μM) K_i	Cat S (μM) K_i	B/K	L/K	S/K
 **9**	0.38	140	3.4	33	368	9	87
 **10**	3.6	>150	16	53	>40	4	15

commercially available 6-APA in four steps following the procedure described by Arnould and Pasquet.[7]

A series of 3,4-disubstituted azetidinones was obtained by removing the Cbz protecting group from the intermediate **1**,[8] and coupling the free amine to various *N*-protected amino acids, using HATU as an activating agent and diisopropylethyl–amine (DIPEA) as a base.[9]

Cat K inhibition and selectivity data are presented in Table 1.[10] Compounds **5**–**8** were tested as a 1:1 mixture of diastereomers. Attempts to synthesize the mixture of the corresponding *trans*-isomers of **5** and **6** have failed. Compound **2**[5] was a potent and non-selective inhibitor of Cat K. Here, we have shown that the use of cyclic P2 elements have a dramatic influence on the selectivity of the compounds, especially against Cat L and Cat S. From the P2 elements investigated, the compound bearing a P2-leucine (**2**) displayed the greatest potency, while those bearing the Ac6 and *cis*-Cy6[11] (**3** and **5**) moieties were the most selective (see Table 1). Since **3** was marginally more selective than **5**, the Ac6 moiety was chosen as a structural element in the synthesis of more potent and selective Cat K inhibitors.[12]

The lack of potency of **7**, compared to **2** and **3**, is probably due to the loss of hydrogen bonding of the P_1–P_2 amide bond with the oxygen of Gly-66, as was reported for several ketone-based Cat K inhibitors that bind to the non-prime side of the active site.[13]

Moreover, preliminary results with the phenoxy group as a substituent in the 4-position of the azetidin-2-one ring (see Table 2), showed that the α (*trans*) orientation is preferred over the β (*cis*) orientation for potency and, to a lesser extent, for selectivity. Compounds **9** and **10** were obtained following synthetic methods described by Singh et al.[5]

In this paper, we have disclosed a novel series of selective 3,4-disubstituted azetidinones based inhibitors of the cysteine protease Cat K. The use of cyclic moieties as P2 elements have proven to be crucial to achieve selectivity over other cathepsins, specially against Cat L and Cat S.

Acknowledgements

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8. **Standard procedure for hydrogenolysis**: To a solution **1** (1 mmol) in ethyl acetate (15 mL), 10% Pd/C (200 mg) was added. The mixture was hydrogenated at 50 psi for 8 h. The catalyst was separated by filtration through a short plug of Celite and the solution of the amine was used for coupling.
9. **Standard procedure for coupling**. To a solution of the corresponding acid (1 mmol) in THF (10 mL), HATU (380 mg, 1 mmol), a solution of the free amine in ethyl acetate (15 mL) and diisopropylethylamine (209 μL, 1.2 mmol) were added at rt. The reaction mixture was stirred for 18 h at rt. The mixture was washed with satd $NaHCO_3$, dried over Na_2SO_4, evaporated and the crude was purified by silica gel column, using a mixture of ethyl acetate/hexane (1:1). Satisfactory spectral data was obtained for all compounds.
10. The enzyme active site concentrations were measured by titration with the either E-64 or an in-house vinyl-sulfone. **Inhibition assays**: Inhibitor potency measurements were performed at room temperature using 96-well kinetic plate readers. Reaction velocities were monitored at varying inhibitor concentrations by following the hydrolysis of aminomethylcoumarin substrates (ex_{355}, em_{460}) as indicated. All substrates were added at a concentration equal to their K_m. Control reactions in the absence of inhibitor were performed in parallel. The K_i apparent (K_i') values were determined by a non-linear least squares regression fit of the experimentally derived data to the Morrison equation for tight-binding inhibitors as described (3) or by least squared regression fit of the Henderson equation for tight-binding inhibitors (4). Enzyme and inhibitor were incubated 30 min prior to initiation of reaction by the addition of substrate. **Cathepsin B**: Enzyme (5.0 nM) was mixed with inhibitor in 50 mM MES or BES (pH 6.0), 2.5 mM DTT, 2.5 mM EDTA, 0.05% Tween 20 and 10% DMSO. The substrate was Z-Phe-Arg-AMC (300 μM). **Cathepsin K**: Enzyme (3.6 nM) was mixed with inhibitor in 50 mM MES (pH 5.5), 2.5 mM DTT, 2.5 mM EDTA, 0.05% Tween-20 and 10% DMSO. The substrate was *Z*-Phe-Arg-AMC (40 μM). **Cathepsin L**: Enzyme (1.3 nM) was mixed with inhibitor in 50 mM MES (pH 5.5), 2.0 mM EDTA, 2 mM DTT, 0.05% Tween-20 and 10% DMSO. The substrate was *Z*-Phe-Arg-AMC (10 μM). **Cathepsin S**: Enzyme (1.0 nM) was mixed with inhibitor in 50 mM MES (pH 6.5), 100 mM NaCl, 2.5 mM EDTA, 2.5 mM 2-mercaptoenthanol, 0.001% bovine serum albumin and 10% DMSO. The substrate was *Z*-Val-Val-AMC.
11. Ac6 stands for alicyclic 1,1-disbustituted cyclohexyl and Cy6 for 1,2-disubstituted cyclohexyl.
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