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Identification of new peptide amides as selective cathepsin L inhibitors: The first step towards selective irreversible inhibitors?

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ABSTRACT

A small library of peptide amides was designed to profile the cathepsin L active site. Within the cathepsin family of cysteine proteases, the first round of selection was on cathepsin L and cathepsin B, and then selected hits were further evaluated for binding to cathepsin K and cathepsin S. Five highly selective sequences with submicromolar affinities towards cathepsin L were identified. An acyloxymethyl ketone warhead was then attached to these sequences. Although these original irreversible inhibitors inactivate cathepsin L, it appears that the nature of the warhead drastically impact the selectivity profile of the resulting covalent inhibitors.

The endopeptidase cathepsin L (CatL) belongs to the papain-like cysteine proteases, which comprise 11 human cysteine cathepsins: cathepsins B, C, H, F, K, L, O, S, V, W and X/Z. In spite of the great similarities in the primary and tertiary structures among the cathepsins, these proteases have distinct enzymatic properties, as reflected in their different substrate specificities. Consequently the cathepsins have different roles in normal and pathological states,[1,2] including cancers.[3–5] Human[6] and animal[7] studies have clearly shown over-expression of CatL in various pathological processes, along with some of these other proteases.[8–10] CatL and CatB have been implicated in several steps of tumorigenesis and cancer progression.[5,11,12] Simultaneously increased protein levels of CatL and CatB have been reported in breast, gastrointestinal, head and neck, and lung cancers, and in glioblastoma and other cancers.[6]

In glioblastoma multiformae, which represent the most invasive stage of glial tumour astrocytomas, CatB has been shown to be relevant for tumour-cell invasion,[13,14] apoptosis[15] and angiogenesis. Multivariate analyses have shown that CatB also has a strong impact on prognosis.[16] In contrast, the role of elevated CatL[17,18] is less clear, although it appears to be related to tumour-cell invasion, and more likely to tumour-cell apoptosis[9,19] and resistance to therapy.[20] A tool for the detection and selective inhibition of the active forms of CatL would help to define its role in cancer progression, as well as in other pathologies.

The papain-like cysteine proteases share highly conserved active sites, with a catalytic dyad that is made up of a key nucleophile (Cys-25) and a general acid (His-159). The superposition of various X-ray structures of these cysteine cathepsins has provided support for only three well-defined substrate-binding sites: S_2, S_1 and $S_{1'}$. Outside of these regions, further sub-sites are not considered to be actual binding sites, but are more appropriately considered to be areas in which the substrate residues find their most favourable binding positions.[1]

Most of the synthetic cysteine protease inhibitors reported to date are covalent inhibitors that exploit this extremely well-defined structural context.[21] Indeed, these inhibitors incorporate a peptide or pseudo-peptide segment in their structure that corresponds to the sequences of good substrates, along with an additional reactive electrophilic moiety, or 'warhead', that can covalently modify the catalytic cysteine[21,22] (e.g., Fig. 1, compound A). Irreversible[23–26] and reversible[27–31] inhibitors are obtained according to the chemical nature of this electrophile. Although a number of potent cysteine protease inhibitors have been described in the literature, achieving selectivity towards only one member of the cathepsin family remains a challenge. In any series of covalent inhibitors, addressing the selectivity for a given target requires the optimisation of both the initial affinity binding step and the subsequent inactivation step. A direct evaluation of libraries of covalent

Abbreviations: AOMK, acyloxymethyl ketone; Cat, cathepsin.

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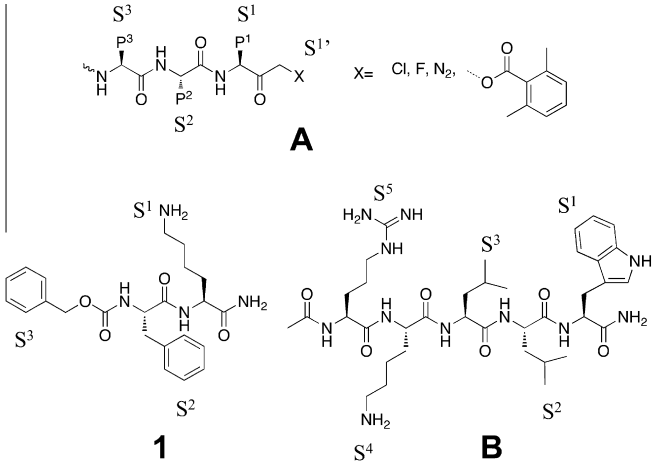
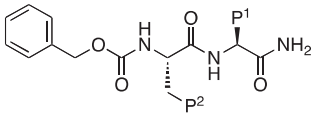


Figure 1. Structures of the covalent inhibitors of the cysteine proteases (A), the peptide amide as a potent CatL inhibitor (B), and the starting structure of the reversible inhibitor, compound **1**.

inhibitors for these targeted enzymes can be envisaged, but access to a large set of compounds often remains difficult to implement, mainly due to the sophisticated chemistry that is used to incorporate the reactive moieties.[32] In contrast, an indirect approach would imply initial profiling of the targeted enzyme active site with peptide or pseudo-peptide sequences, and then providing the selected segments with the necessary warhead. Such an approach represents a major advantage for easy and rapid screening of a large set of compounds with high chemical diversity using standard peptide chemistry. However, this strategy does not allow the simultaneous profiling of primed and non-primed regions. Such an approach was recently reported for CatK, which led to the identification of a relatively selective irreversible CatK inhibitor.[33] In this context, we have here evaluated the feasibility of such a strategy to access selective covalent inhibitors of CatL.

Compared to the approach described by Choe et al.[33] that relied on the screening of fluorogenic substrates, we evaluated a library of simple di-peptide and tri-peptide amides. This approach is supported by the work of Brinker et al.[34] that led to the identification of a peptide amide as a potent CatL inhibitor (Fig. 1, compound B; K_i 130 nM) that can interact within the non-primed region of the active site without being cleaved at its C-terminal position. As a starting point, we re-explored and optimised the affinity and selectivity profile of the CbzPheLys sequence, a motif that is classically encountered in irreversible inhibitors and series of activity-based probes.[21,24] Thus, from the pseudo-peptide amide **1** (Fig. 1), chemical diversity was introduced alternatively at each position (P^1, P^2 and P^3). Several substrates and inhibitors of cysteine cathepsins have a basic residue in the P^1 position.[24,33,35,36] To evaluate the precise impact of a P^1 lysine on the affinity and selectivity profile towards CatL, basic residues that varied in length and/or flexibility, and also uncharged residues, were tested in the P^1 position (Table 1, compounds **2**–**12**). The substrate specificity profiles for CatL show a preference for bulky hydrophobic and aromatic residues in the P^2 position.[24,33–35,37] On the assumption that a substituted phenyl moiety would be better tolerated within the S_2 sub-site of CatL compared to other cysteine proteases, several aromatic side chains that varied in length and substitution were introduced in the P^2 position (Table 1, compounds **13**–**23**). Finally, as already mentioned, the S^3 and S^4 substrate-binding sites of CatL are not considered to be actual binding sites, which suggests that they are susceptible to accommodate a large panel of various residues that might be either hydrophobic or basic.[35,38] The carboxybenzoyl moiety was therefore replaced by various acyl moieties, and by randomly selected amino acids (Table 2, compounds **24**–**45**). This small library

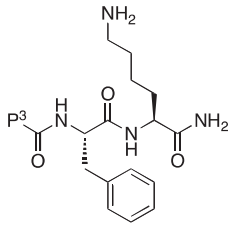
Table 1
Effects of the P^1 and P^2 substituent on affinity towards CatL and CatB



	P_1	P_2	K_i[a] (μM) CatL	K_i[a] (μM) CatB	F_B[b]
1	$-(CH_2)_4-NH_2$	$-(C_6H_5)$	4.2	3406	**810**
2	$-(CH_2)_4-NH-CH_3$	$-(C_6H_5)$	2.6	1547	**595**
3	$-(CH_2)_3-CH(OH)-NH_2$	$-(C_6H_5)$	27	1734	64
4	$-(CH_2)_3-NH_2$	$-(C_6H_5)$	3.5	2460	**703**
5	$-NH_2$	$-(C_6H_5)$	28	ND	ND[c]
6	$-(CH_2)-(4\text{-}NH_2-C_6H_4)$	$-(C_6H_5)$	26	2787	**107**
7	$-(CH_2)_3-NH-(C{=}NH)-NH_2$	$-(C_6H_5)$	28	349	12
8	$-(CH_2)$-1*H*-Imidazol	$-(C_6H_5)$	14	ND	ND[c]
9	$-(CH_2)_2-S-CH_3$	$-(C_6H_5)$	64	7096	**111**
10	$-(CH_2)-(CH)-(CH_3)_2$	$-(C_6H_5)$	14	4341	**310**
11	$-(CH)-(CH_3)_2$	$-(C_6H_5)$	17	ND	ND[c]
12	$-CH_3$	$-(C_6H_5)$	16	ND	ND[c]
13	$-(CH_2)_4-NH_2$	$-(C_6H_4)-p\text{-}(C_6H_5)$	2.4	77	32
14	$-(CH_2)_4-NH_2$	$-(C_6H_4)-p\text{-}(3\text{-}NH_2-C_6H_4)$	1.8	38	21
15	$-(CH_2)_4-NH_2$	$-(C_6H_4)-p\text{-}(3\text{-}Cl-C_6H_4)$	1.7	58	34
16	$-(CH_2)_4-NH_2$	$-(C_6H_4)-p\text{-}(3\text{-}CH_3-C_6H_4)$	2.5	389	**156**
17	$-(CH_2)_4-NH_2$	$-(C_6H_4)-p\text{-}(2\text{-}CH_3-C_6H_4)$	23	245	11
18	$-(CH_2)_4-NH_2$	$-(C_6H_4)-p\text{-}(4\text{-}CH_3-C_6H_4)$	1.7	113	66
19	$-(CH_2)_4-NH_2$	$-(C_6H_4)-p\text{-}(C_6H_4)-p\text{-}(C_6H_5)$	2.6	74	28
20	$-(CH_2)_4-NH_2$	$-(C_6H_4)-p$-(2-Thiophen)	0.12	39	**325**
21	$-(CH_2)_4-NH_2$	1*H*-Imidazol	3.1	56	18
22	$-(CH_2)_4-NH_2$	1*H*-Indol	4.2	100	24
23	$-(CH_2)_4-NH_2$	$-(4\text{-}OH-C_6H_4)$	1.4	135	96

Bold values refer to selectivity factors of 3 orders of magnitude by comparison with those of only 2 orders or less.
[a] K_i values were determined at a minimum of three concentrations of inhibitors (measured in duplicates).
[b] $F_B = K_i(\text{CatB})/K_i(\text{CatL})$.
[c] ND, not determined.

Table 2
Effects of the P^3 substituent on the affinity towards CatL and CatB



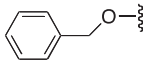
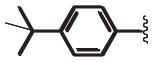
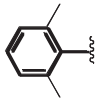
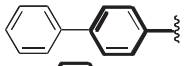
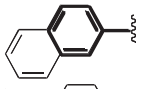
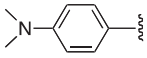
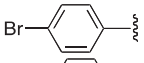
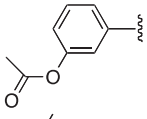
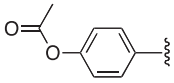
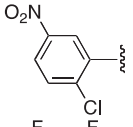
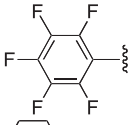
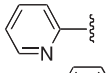
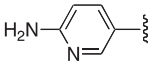
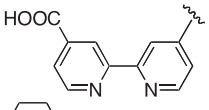
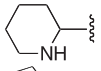
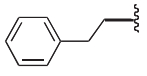
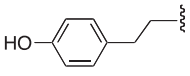
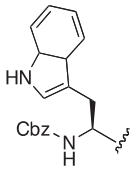
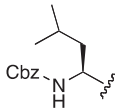
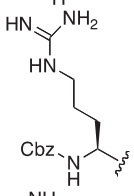
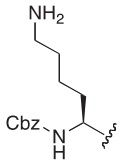
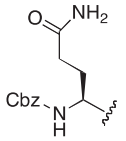
	P_3	K_i[a] (μM) CatL	K_i[a] (μM) CatB	F_B[b]
1		4.2	3406	**811**
24		1	312	**312**
25		20	3387	**169**
26		9	84	9
27		1	301	**301**
28		2.9	26	9
29		2.3	261	**113**
30		2.4	1020	**425**
31		2.6	706	**272**
32		2.7	1757	**651**
33		6.8	134	20
34		5.5	80	15
35		5	148	30
36		4.6	860	**187**
37		6.1	51	8.4
38		7.8	623	80
39		3.2	2035	**636**
40		2.5	135	54

Table 2 (*continued*)

	P_3	K_i[a] (μM) CatL	K_i[a] (μM) CatB	F_B[b]
41		0.39	835	**2141**
42		0.45	1185	**2633**
43		0.74	1303	**1761**
44		0.89	1494	**1679**
45		7	3545	**506**

Bold values refer to selectivity factors of 3 orders of magnitude by comparison with those of only 2 orders or less.

[a] K_i values were determined at a minimum of three concentration of inhibitors (measured in duplicates).

[b] $F_B = K_i(\text{CatB})/K_i(\text{CatL})$.

of pseudo di-peptides and tri-peptides was synthesised on rink amide lanterns (Mimotopes®) using standard Fmoc-based solid-phase peptide synthesis. To access differently substituted biphenyl-like side chains in the P^2 position, post-modification was carried out on the 4-iodo-phenyl side-chain on a solid support, using Suzuki coupling conditions and under microwave irradiation[37] (see Supplementary data for details). The peptides were then individually cleaved from the lanterns using trifluoroacetic acid/triisopropylsilane/water (95/2.5/2.5), and purified by reverse-phase HPLC. All of the peptides were dissolved in dimethylsulphoxide as trifluoroacetic acid salts and characterised by ion-spray mass spectrometry, with their purities assessed by analytical HPLC (see Supplementary data, Table S1). The concentration of each of the stock solutions was determined by amino-acid measurement. In the primary screening, these peptide amides were evaluated in kinetic studies, using recombinant CatL and CatB (see Supplementary data, experimental procedures). For these compounds, a selectivity factor F_B corresponding to $K_i(\text{CatB})/K_i(\text{CatL})$ was defined (Tables 1 and 2). The selected inhibitors that showed significant selectivity (F_B >1000) and affinity profiles towards CatL were further tested on two other cysteine cathepsins, CatK and CatS. This led the determination of factors F_S and F_K corresponding to $K_i(\text{CatS})/K_i(\text{CatL})$ and $K_i(\text{CatK})/K_i(\text{CatL})$, respectively (Table 3).

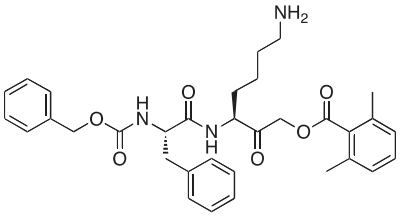
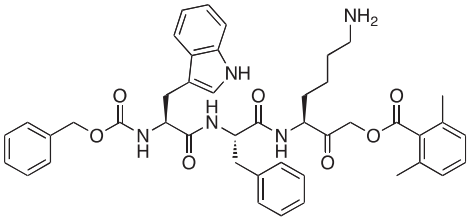
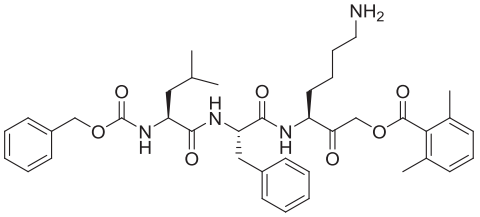
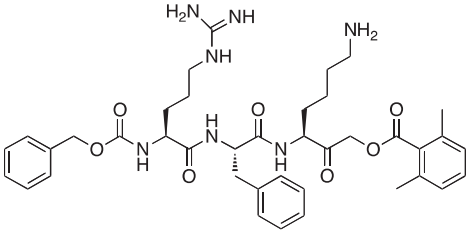
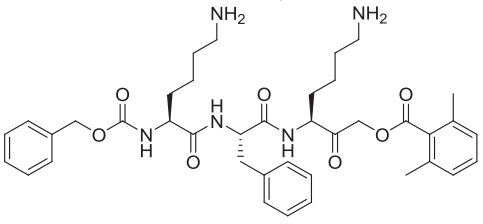
Among all of the compounds with a variable P^1 position (Table 1, compounds **2**–**12**), only compounds **2** and **4** showed potency and selectivity comparable to peptide **1** towards CatL. These two compounds have an *N*-methylated lysine side-chain and a shorter side-chain in the P_1 position, respectively. A long and flexible basic side chain in the P^1 position was required to maintain substantial affin-

Table 3
Selectivity and affinity profiles for compounds **1**, **20**, **41**–**44** towards CatL, CatB, CatS and CatK

	K_i[a] (μM) CatL	K_i[a] (μM) CatB	K_i[a] (μM) CatS	K_i[a] (μM) CatK	F_B[b]	F_S[c]	F_K[d]
1	4.2	3406	56	47	**811**	13	11
20	0.12	39	28	24	**325**	**233**	**200**
41	0.39	835	20	639	**2141**	51	**1638**
42	0.45	1185	12	213	**2633**	27	**473**
43	0.74	1303	8.5	147	**1761**	11	**198**
44	0.89	1494	41.1	284	**1679**	46	**319**

Bold values refer to selectivity factors of 3 orders of magnitude by comparison with those of only 2 orders or less.
[a] K_i values were determined from a minimum of four concentration of inhibitors (measured in duplicates).
[b] $F_B = K_i(\text{CatB})/K_i(\text{CatL})$.
[c] $F_S = K_i(\text{CatS})/K_i(\text{CatL})$.
[d] $F_K = K_i(\text{CatK})/K_i(\text{CatL})$.

Table 4
Association constants (k_{ass}) of irreversible inhibitors **A-AOMK**, **46**–**49** for CatL and CatB

		k_{ass}[a] (s^{-1} M^{-1}) CatL	k_{ass}[a] (s^{-1} M^{-1}) CatB	k_{ass} CatL/k_{ass} CatB
A-AOMK		2.96×10^5	0.38×10^5	7.8
46		3.92×10^5	0.57×10^5	6.9
47		2.69×10^5	1.36×10^5	2.0
48		8.75×10^5	0.76×10^5	11.5
49		2.52×10^5	8.22×10^5	0.3

[a] k_{ass} were determined by the progression of the curve analysis under the pseudo-first-order conditions, with at least 10-fold molar excess of the inhibitor over the enzyme.

ity for CatL, as illustrated by the loss of potency for compounds **5** and **6**, which have a simple amino function and an aniline-like side-chain in the P^1 position, respectively. Compound **7** has a P_1 arginine, and it did not show any selectivity for the CatL active site over that of CatB. These data are in agreement with the literature, where it has been reported that a P^1 arginine residue promotes the activity of all cysteine proteases.[33] Although uncharged residues can interact with the CatL S^1 sub-site, the corresponding peptides (compounds **9**–**12**) were less potent towards CatL. Overall, these data confirm those from the literature: a free lysine group at the

P^1 position is necessary to maintain a favourable affinity and selectivity profile towards CatL.[24]

With the exception of compound **17**, all of the peptides with a P^2 biphenyl-like side chain (Table 1, compounds **13**–**18**) were as potent as compound **1** towards CatL, and were significantly more potent inhibitors of CatB, thus reducing their selectivity factors with regard to CatL. The same occurred for the compounds with a P^2 triphenyl side chain (compound **19**) and with other aromatic moieties (compounds **21**–**23**). Interestingly, compound **20**, which has a P_2 phenyl thiophen side-chain, showed a significant, 35-fold increase in affinity for CatL compared to reference peptide **1**, with a K_i of 120 nM. Although compound **20** also showed a 87-fold gain in the potency for CatB, the selectivity factor remained largely in favour of CatL (F_B = 325). Overall, compared to peptide **1** with a P^2 phenyl moiety, none of the modifications introduced at the P^2 position resulted in peptides with any significantly increased selectivity for CatL.

In contrast, the P^3 and P^4 positions appeared to be critical for the modulation of both the inhibitor affinity and selectivity towards CatL and CatB (Table 2, compounds **24**–**45**). The introduction of various acyl moieties in the P^3 position (compounds **24**–**40**) only had a moderate impact on the inhibitor potency for CatL. In contrast, the affinity of these peptides towards CatB ranged from millimolar concentrations to 26 μM for the most potent, as compound **28**. On the other hand, the inclusion of *N*-carboxybenzoyl protected amino acids in the P^3 position, such as tryptophane in compound **41**, leucine in compound **42**, arginine in compound **43** and lysine in compound **44**, led to potent inhibitors with sub-micromolar affinities for CatL. Surprisingly, these modifications had almost no impact on their affinities for CatB. This led to the identification of four compounds (**41**–**44**) with selectivity factors F_B that showed three orders of magnitude in their selectivity for CatL (Table 2).

In spite of the topologically comparable non-primed regions between the CatL and CatB active sites, we successfully identified five original short peptides that can interact potently with CatL while maintaining an excellent selectivity profile towards CatL (compounds **20**, **41**–**44**). The affinities of these compounds towards two other members of the papain-family proteases, CatS and CatK, were also determined, as shown in Table 3. Interestingly, despite the canonical cathepsin cysteine specificity for aromatic residues in the P^2 position, it appeared that elongating the P_2 phenyl moiety with a thiophen heterocycle did not have the same effects on CatL and CatB. There was a marked gain of affinity for CatL compared to CatK and CatS, where there were almost no effects on affinity (Table 3, compound **20**). This pseudo-peptide thus shows favourable affinity and selectivity for CatL with selectivity factors of two orders of magnitude over CatB, CatS and CatK. Comparisons of their S^2 regions did not highlight any marked topological differences that might explain these data (Supplementary data, Fig. S1). Compared to peptide **1**, an additional *N*-carboxybenzoyl-protected amino acid in the P^3 position led to compounds with slightly improved potency toward CatS, but which showed lower affinity for CatK (Table 3, compounds **41**–**44**). When analysing the topological differences between the S^3 regions of CatL, CatB, CatS and CatK, it appeared that CatK, and to a lesser extent CatB, has a narrower S^3 sub-site compared to CatL and CatS (Supplementary data, Fig. S1). These observations suggested that a bulky P^3 substituent would not correctly enter the S^3 sub-sites in CatK and CatB, which might explain the poor affinity of compounds **41**–**44** towards CatK and CatB. However, although they show poor selectivity factors with regard to CatS, compounds **41**–**44** retained their selectivity for CatL versus CatK and CatB.

To follow the idea of progressing from reversible peptides to covalent inhibitors with optimised affinity and selectivity toward CatL, only the compounds displaying selectivity factors F_B of three orders of magnitude (**41**–**44**) were selected for further modification. It was hypothesised that only sequences with a marked gain of selectivity compared to the sequence of reference **1** would significantly impact the selectivity profile of the resulting irreversible inhibitors. The acyloxymethyl ketone (AOMK) warhead was chosen as a quiescent reactive moiety. Compounds containing AOMK were first reported by Krantz et al.,[39] who claimed that they are clinically useful inhibitors of cysteine cathepsins as they are stable in human serum, non-toxic for cells, and soluble and cell permeant.[21,24] Four covalent irreversible inhibitors, AOMK derivatives of compounds **41**–**44**, compounds **46**–**49** were thus synthesised as previously described.[40] It appeared that, whatever was the peptide sequence, the resulting AOMK derivatives inactivate both CatL and CatB in a one-step process. In such situation, affinity binding constants (K_i) and inactivation rate (kinact) were not accessible as the adsorptive EI complex is kinetically irrelevant. Thus only association constants (k_{ass}) towards CatL and CatB can be determined (Table 4 and see Supplementary data, Fig. S2 for details). Hence no correlation between the reversible and irreversible inhibitors can be established in regards to their selectivity profiles towards CatB and CatL. Our data clearly highlight the major influence of the warhead on the selectivity profile. Beyond the steric hindrance of the AOMK moiety, which will impact necessarily on the inhibitor binding, a warhead with a relatively high intrinsic reactivity will tend to react equally with the conserved nucleophile that adopts a similar orientation within all the cysteine protease active sites. Consequently, this might have unfavourable effects on the selectivity profiles towards these enzymes. Significantly and despite similar factor of selectivity F_B for compounds **43** and **44** (Table 3), the resulting AOMK inhibitors **48** and **49**, respectively, displayed very different profiles of selectivity (Table 4). Our data clearly suggest that the selected warhead was not appropriate to draw a general conclusion on the selectivity changed between the peptides and their AOMK analogues.

In the present study, we have identified original di-peptide and tri-peptide motifs as potent and selective reversible CatL inhibitors. These peptide sequences might be useful for the future development of covalent inhibitors directed towards CatL. However, further covalent reversible and irreversible warheads have to be screened to evaluate the precise impact of such peptide sequence optimisation. Finally, since most of the cathepsins are intracellular proteases, the real selectivity profile of inhibitors has to be assessed in cellular assays. Selective inhibitors against purified cathepsins might display different and in some cases diminished selectivity in cells due to lysosomotropism, which is especially true for peptide sequence containing basic residue such as compounds **43** and **44**.[41]

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Supplementary data

Supplementary data (the synthesis of the di-peptides and tri-peptides and the acyloxymethyl ketones, the in vitro enzyme assays, the analytical characterisation of all of the synthesised molecules, and the superimposition of the cathepsin active sites) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.041.

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