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Inhibition of human β-tryptase by Bowman–Birk inhibitor derived peptides: creation of a new tri-functional inhibitor

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Abstract—Bowman–Birk inhibitor proteins (BBIs), which are potent inhibitors of chymotrypsin-like proteases, do not inhibit human β-tryptase despite this protein having a chymotrypsin-like fold. We have reported previously that, in contrast, BBI-derived peptides (whose sequences incorporate the solvent exposed reactive site loop motif) are able to inhibit human β-tryptase. This is due to their small size, which allows them to access the restricted active site(s) of tryptase, which has an unusual tetrameric arrangement with four active sites flanking a central pore. In this paper, we have examined the possibility of creating additional interactions within this pore by adding extensions to the BBI–peptide motif. We have taken the core disulfide-bridged sequence SCTKSIPPQCY and examined a series of extensions, at both the C- and N-termini, that bear a second positively charged Lys residue at their end. The aim was to construct inhibitors that could make additional interactions in tryptase by spanning the gap between adjacent active sites in the enzyme, producing a double-headed inhibitor; a positively charged group was used as the dominant specificity of this enzyme is for a positively charged P1 residue. Both N- and C-terminal extensions are found to produce inhibitors of much increased potency, with a strong dependence of potency on chain length. Moreover, it was found that the C- and N-terminal extensions were able to synergise, with their combination on the same peptide producing an even better inhibitor with a potency 10^4-fold greater than the original sequence. We suggest that the C- and N-terminal extensions are picking up interactions with separate additional sites on the tryptase, making the doubly extended BBI peptide a tri-functional tryptase inhibitor.

1. Introduction

Human tryptase (E.C.3.4.21.59), which has a trypsin-like specificity,[1] is the main protein in most human mast cells and constitutes the 20–25% of the total content of the cell.[2] There are eight different isoenzymes of β-tryptase, which is predominantly expressed in both lung and skin mast cells.[3] Recently, the complex between β-tryptase and 4-amidinophenylpyruvic acid (APPA), an inhibitor with a K_i of 0.71 mM,[4] has been solved by X-ray crystallography.[5]

Tryptase is involved in inflammatory and allergic disorders, among them asthma, rhinitis, multiple sclerosis, psoriasis, interstitial cystitis and rheumatoid arthritis. Most studies have focused on asthma and even though the substrates for this enzyme have not yet been fully characterised and understood,[6] it seems that they are the peptides critical for the maintenance of the smooth muscle tone of the airways.[7,8] Once the mast cell is exposed to an allergenic factor, many mediators are released into the extracellular environment[9] including, along with other proteases, tryptase together with heparin, its stabilising agent.[10]

Tryptase is an atypical serine protease as its active form is a tetramer that is formed of four almost identical monomers.[5,11] Although this subunit organisation is unique to tryptase, the individual active sites are structurally very similar to other serine proteases and show the characteristic catalytic triad. The organisation of the subunits is represented in Figure 1,[5] where there are two pairs of identical monomers A–C (yellow) and B–D (green). In red are highlighted the four molecules of APPA that bind to the active sites of the enzyme. The

Abbreviations: BBI, Bowman–Birk inhibitor; APPA, 4-amidinophenylpyruvic acid; LDTI, Leech-derived tryptase inhibitor.

Keywords: Human β-tryptase; Bowman–Birk inhibitor; Peptide synthesis; Double-headed inhibitors; Leech-derived tryptase inhibitor.

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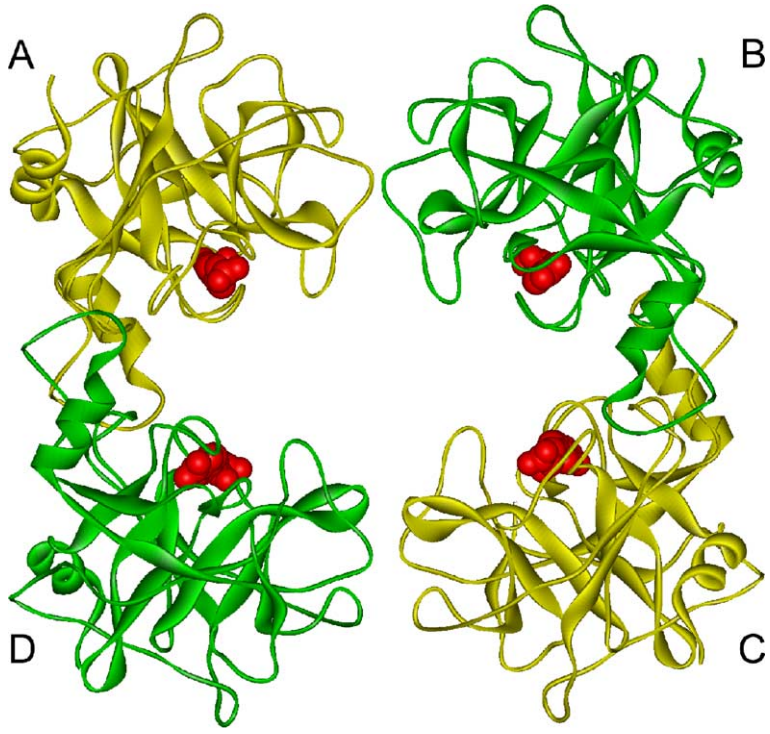

Figure 1. Human β-tryptase structure.[5,11] The four monomers are arbitrarily named A, B, C and D. The two identical pairs of monomers are highlighted in yellow (A–C) and green (B–D). The 4-amidinophenylpyruvic acid is shown in red.

two pairs of monomers differ in their contact regions where the conformation of Tyr75 causes a break of the local twofold symmetry.[5,11] Each monomer contains an active site and the four active sites face a central ellipsoidal pore. The major axis measures ~50 Å and the minor one ~30 Å. The distance between two adjacent active sites are of either 20 Å (A–D and B–C) or 40 Å (A–B and C–D), respectively. The central pore is partially occluded by the 147 loops.

To stabilise itself in the noncovalent tetrameric active form, the enzyme requires binding to heparin proteoglycans along the A–D and B–C axes, through a number of positively charged residues.[5] Without heparin or acidic polysaccharides (and also at high salt concentrations in vitro) tryptase readily evolves into a formally inactive monomeric form.[10,12]

There is much interest in obtaining inhibitors of tryptase, and the unusual structure has been used to help build bifunctional inhibitors that show greater specificity. Many inhibitors have already been synthesised, most of which contain either a benzamidino[4,6,9,13,14] or a guanidino moiety,[15–20] useful to the molecular recognition by the enzyme.

The only known natural inhibitor of tryptase has been, for almost 20 years, Leech-derived tryptase inhibitor (LDTI[21]). This is a small protein (46 amino residues) that shows potent inhibition of half the active sites (K_i 1.4 nM) but weaker inhibition for the remaining sites against human mast cell tryptase.[3] The binding loop responsible for the protease inhibition seems to be Cys6-Lys11, with Lys8 as the primary determinant of inhibitor specificity.[22] Two new tryptase inhibitors, cyclic pentapeptides cyclotheonamides E4 and E5,[23] were recently isolated from a marine sponge of the genus *Ircinia*. Cyclotheonamides are cyclic pentapeptides that contain two unusual aminoacids, α-ketohomoarginine and vinylogous tyrosine. The biological activity towards serine proteases is due to the presence of the α-keto group in the arginine residue. Apart from these oligopeptides, tryptase is not inhibited by other potent trypsin inhibitors such as Bovine pancreatic trypsin inhibitor.[24,25]

To the best of our knowledge, the only synthetic peptidic inhibitors of β-tryptase that have been reported are peptidyl arginals[26] and ones based on the Bowman–Birk inhibitor (BBI) structure.[27] BBI peptides are short (9–11 residue) sequences that retain most of the activity of the parent BBI[28] as well as the characteristic canonical protease inhibitor structure.[29] The reason why these peptides are able to inhibit tryptase while the BBI protein does not is thought to be simply a question of size—the BBI protein is not able to access the active site whereas the BBI peptide can fit within the central pore. Unlike LDTI, inhibition by BBI peptide shows no evidence for nonequivalent binding sites, suggesting that the BBI peptide is equally able to access all sites of the tryptase.[27]

2. Results and discussion

The sequence SCTKSIPPQCY (**1**) is a BBI-derived small peptide that encapsulates one of reactive site loops from the protein. The two cysteines are joined via a disulfide bridge that, together with a *cis*-amide bond at

the first proline and a hydrogen bond network, guarantees the formation of an anti-parallel β-sheet conformation, typical of this structure.[29] This sequence has a lysine residue at P_1; changes at this location are known to alter the primary specificity of the inhibitor.[30] Previously we found that peptide **1** is a human β-tryptase inhibitor (K_i = 31.5 μM) and this was chosen as starting sequence for further modifications.

Peptides **2**–**10** and **15**–**26** were synthesised with the aim of realising better inhibitors by virtue of allowing additional interactions. Bivalent inhibitors that span adjacent active sites have proved successful tryptase inhibitors.[9,13,14,16,18,26,31] As the S_1 pocket of tryptase binds basic residues, we elected to introduce a second Lys residue at the N- or C-terminal end of the peptide. These were spaced, respectively, from the original Ser (P_4) or Tyr (P_7) by a linear chain mainly formed by CH_2 units and containing no more than three amide bonds. Due to the distance of the closest active sites (~20 Å), chains in the length range 5–25 Å were chosen, this corresponding to a Lys (P_1)–Lys distance of roughly 16–36 Å.

2.1. Effects of C-terminal extension

A series of inhibitors was prepared incorporating a positively charged lysine spaced at various distances from the C-terminal end of the BBI peptide **1**. For each of the peptides we measured the K_i value for inhibition of tryptase and trypsin and their inhibition constants are summarised in Table 1. Within this group of peptides, it is found that there is a marked improvement in tryptase inhibition when the additional lysine is present. Additionally, there is a minimum in K_i when the C-terminal lysine is approximately 19–21 Å away from the P_1 residue (Fig. 2). The enhancement in binding is specific for tryptase; trypsin binding is largely unaffected by these additional C-terminal sequences. The improvement due to the additional positive charge can be demonstrated by substituting the C-extension lysine for norleucine (compare **4/13**, **5/14**), which also raises the K_i to be comparable to that of the starting peptide **1** (Table 2). Table 2 also records the effect of replacing the terminal carboxyl group with a C-terminal amide (**4/11**, **5/12**). In each case there is an improvement in

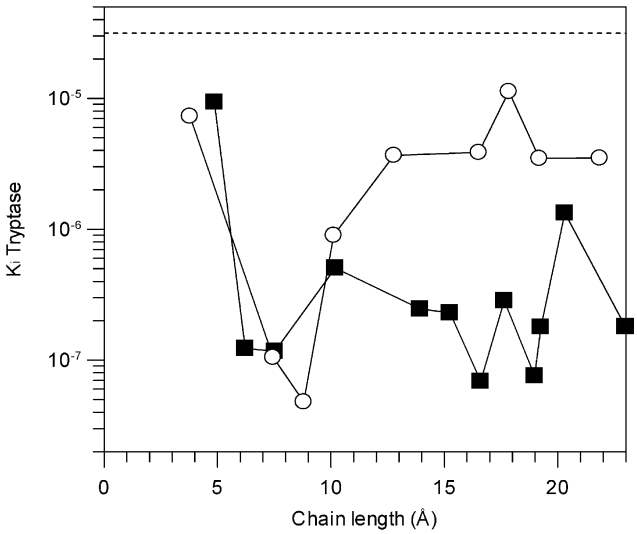

Figure 2. The effect of spacer chain length (Å) on tryptase K_i. The results are shown for BBI peptides bearing an additional positively charged residue at the N-terminal (■) or C-terminal (○) end. The chain lengths are calculated for fully extended conformations and refer to the maximal distance between the carboxyl groups of the P1 lysine and the N-terminal/C-terminal lysine residues. The K_i value for the BBI–peptide sequence lacking any additional positively charged group is shown by the dashed line.

binding from the amide variant. It is possible that this is due to the negatively charged carboxylate making unfavourable interactions with the protein, or because of intramolecular interactions with the adjacent lysine side chain. The inhibition of trypsin is found to be largely unaffected by any of these changes. Inhibitor **4** and its amide derivative **11** are the most potent of this C-terminal series and have a K_i of 48 and 11 nM for tryptase, respectively; these are three orders of magnitude lower than that of peptide **1**, which lacks the C-terminal extension. For all of the peptides within this series we found that the inhibition data for tryptase were consistent with equivalent binding sites. It is not possible, from these activity measurements, to quantify the number of sites but we think it is likely that the potent binding by inhibitors **4** or **11** is due to them being able to bridge between adjacent active sites of the tryptase. Such rationalisation is consistent with other work on bifunctional tryptase inhibitors[9,13,14,16,18,26,31] and

Table 1. Inhibition of β-tryptase and trypsin by BBI-derived peptides: effect of differing C-terminal extensions

	Sequence	P1–lysine distance (Å)	K_i tryptase (μM)	K_i trypsin (μM)
1	SCTKSIPPQCY		31.5	0.047
2	SCTKSIPPQCYKG	16.7	7.3	0.025
3	SCTKSIPPQCYGKG	20.4	0.11	0.013
4	SCTKSIPPQCYβAlaKG	21.7	0.048	0.036
5	SCTKSIPPQCYAbuKG	23.0	0.90	0.008
6	SCTKSIPPQCYεAhxKG	25.7	3.67	0.008
7	SCTKSIPPQCYGεAhxKG	29.4	3.87	0.046
8	SCTKSIPPQCYεAhxβAlaKG	30.7	11.3	0.069
9	SCTKSIPPQCYεAhxAbuKG	32.1	3.48	0.044
10	SCTKSIPPQCYεAhxεAhxKG	34.8	3.50	0.028

Peptides **2**–**10** have a lysine residue positioned adjacent to the C-terminal end. The chain length (Å) is calculated between the carboxyl groups of the P1 lysine and the C-terminal lysine residues and refers to a fully extended conformation. All peptides incorporate a disulfide bridge linking the two cysteines. Errors in K_i are typically ±10%.

Table 2. Inhibition of β-tryptase and trypsin by BBI-derived peptides: role of charge in the C-terminal extensions

	Sequence	K_i tryptase (μM)	K_i trypsin (μM)
4	SCTKSIPPQCYβAlaKG	0.048	0.036
5	SCTKSIPPQCYγAbuKG	0.090	0.008
11	SCTKSIPPQCYβAlaKG-NH_2	0.011	0.021
12	SCTKSIPPQCYγAbuKG-NH_2	0.029	0.034
13	SCTKSIPPQCYβAlaNleG	4.97	0.027
14	SCTKSIPPQCYγAbuNleG	13.9	0.035

All peptides incorporate a disulfide bridge linking the two cysteines. Errors in K_i are typically ±10%.

would explain why the effect is only found with tryptase and not with trypsin. The distance between the P_1 lysine and the C-extension lysine in the most active peptides is consistent with the separation between adjacent active sites in tryptase. All the peptides studied included a glycine residue following the lysine. We have examined whether there is evidence for any peptide bond cleavage in these inhibitors and can detect no discernible hydrolysis under the conditions of our assay (results not shown). The peptides all appear to act as simple competitive inhibitors.

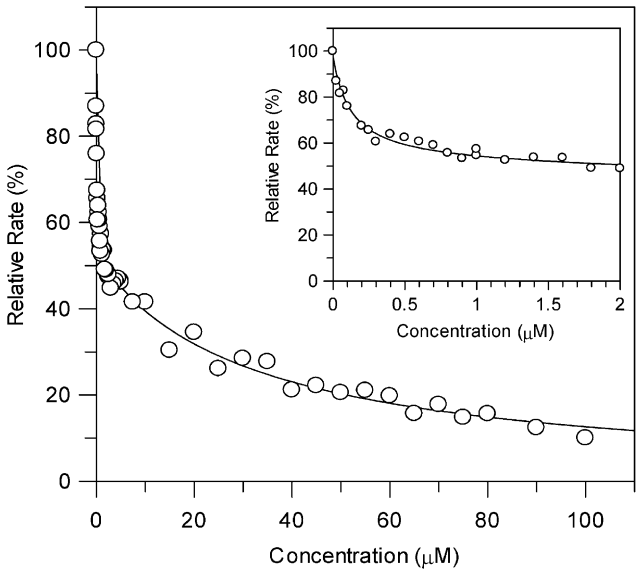

Figure 3. Bimodal tryptase inhibition. Inhibition of tryptase by peptide **18** (inset, expanded concentration range) shows a characteristic bimodal shape. The data were fitted to Eq. 2 to obtain two separate inhibition constants.

2.2. Effects of N-terminal extension

The results of adding an additional lysine onto the N-terminal end of the peptide are shown in Table 3. The effects are, in broad terms, analogous to those for C-terminal extensions as it is found that there is a marked improvement in tryptase inhibition (lower K_i) when an additional lysine is present.

When the inhibition data are examined more closely, however, there is a significant difference between the inhibition kinetics of most peptides of Table 3 and those that include a C-terminal extension. While the C-terminal series show simple inhibition data, many of the peptides in Table 3 show biphasic plots. Figure 3 illustrates inhibition of tryptase by peptide **18**. The data are inconsistent with a single binding mode, but are well fitted if two sites of unequal affinity are considered. In this respect, the data are similar to those produced by LDTI, which shows two equivalent tight binding sites plus two further weak sites.[3] For peptide **18** we also find that 50% of the rate is inhibited strongly while the remaining activity is inhibited almost 100-fold more poorly. Such behaviour is strongly reminiscent of LDTI, and suggests a similar mode of action. However, the proportion of activity inhibited strongly is found to vary amongst the series of peptides in Table 3. In the absence of structural data it is only possible to speculate on the implications of these data for binding. If we assume that each of the four tryptase sites shows identical activity, these data

Table 3. Inhibition of β-tryptase and trypsin by BBI-derived peptides: effect of differing N-terminal extensions

	Sequence	P1–lysine distance (Å)	K_i tryptase (μM)	K_i(2) tryptase (μM) [%]	K_i trypsin (μM)
1	SCTKSIPPQCY		31.5	—[a]	0.047
15	KGSCTKSIPPQCY	17.1	9.39	—[a]	0.087
16	KβAlaSCTKSIPPQCY	18.5	0.12	58.9 [59.9%]	0.016
17	KγAbuSCTKSIPPQCY	19.8	0.12	52.5 [49.0%]	0.016
18	KεAhxSCTKSIPPQCY	22.5	0.51	35.2 [49.4%]	0.018
19	KεAhxGSCTKSIPPQCY	26.2	0.24	61.5 [34%]	0.017
20	KεAhxβAlaSCTKSIPPQCY	27.5	0.23	269 [21.5%]	0.022
21	KεAhxγAbuSCTKSIPPQCY	28.9	0.069	—[a]	0.024
22	KεAhxεAhxSCTKSIPPQCY	31.5	0.29	43.2 [9.4%]	0.039
23	KεAhxGGSCTKSIPPQCY	29.9	0.076	74 [6.0%]	0.022
24	KεAhxGβAlaSCTKSIPPQCY	31.2	0.18	342 [21.6%]	0.026
25	KεAhxGγAbuSCTKSIPPQCY	32.6	1.33	—[a]	0.020
26	KεAhxGεAhxSCTKSIPPQCY	35.2	0.18	69.2 [12.6%]	0.019

Peptides **15**–**26** have a lysine residue positioned at the N-terminal end. The chain length (Å) is calculated between the carboxyl groups of the P1 lysine and the C-terminal lysine residues and refers to a fully extended conformation. Where indicated, the inhibition behaviour of the peptides with tryptase shows bimodal inhibition with the data being characterised by a tight and a weak binding interaction. For the biphasic data, a second K_i value is shown together with an indication of the proportion of the data that are represented by the weaker binding. All peptides incorporate a disulfide bridge linking the two cysteines. Errors in K_i are typically ±10%.

[a] Data are consistent with a single class of binding site only.

suggest that, following binding of the inhibitors into the restricted active site clefts of tryptase, binding to subsequent site(s) is hindered and so shows weaker affinity. For the inhibitors where 50% of activity is inhibited strongly, the most likely explanation is that once half the sites are occupied it is more difficult for the final two sites to be taken up. The peptides with larger N-terminal extensions are generally found to show higher proportions of tight binding sites. It is possible that their greater bulk allows them to prevent substrate access more effectively.

A logarithmic plot of the first (lowest) K_i versus N-terminal and P1 Lys residues distance reveals a broad minimum when the chain length is approximately 20–30 Å (Fig. 2). The most potent inhibition is given by peptide **21** ($K_i = 69$ nM), which has an approximate maximum distance of 28.9 Å between the lysines, and which improves on peptide **1** by over 200-fold in terms of K_i value. In contrast, there is no equivalent trend in the inhibition of trypsin, the K_i values for which are essentially unaffected by the N-terminal additions.

As further evidence that the positive charge of the terminal lysine side chain is responsible for the enhanced interaction, Table 4 shows the effect of N-acetylation and of replacing the terminal lysine residue with a neutral norleucine. Peptide **28** ($K_i = 10.3$ μM) differs from peptide **22** ($K_i = 0.29$ μM) by a Lys→Nle substitution, in effect deleting the ε-amino group of the terminal lysine. In the absence of this charged group, the peptide shows no enhanced interaction with tryptase and has a K_i value that is comparable to the starting peptide **1**. Interestingly, this peptide also does not display biphasic inhibition, further implicating the N-terminal lysine in the complex inhibition. Peptide **27** ($K_i = 0.31$ μM) differs from peptide **22** as it is αN-acetylated. These two peptides have very similar inhibition constants. Analogous results are obtained when peptide **23** ($K_i = 0.076$ μM) is modified by either εNH_2 deletion (**30**, $K_i = 0.64$ μM) or αNH_2 acetylation of the N-terminal lysine (**29**, $K_i = 0.15$ μM).Together, these results suggest that it is the ε-amino group that is responsible for the enhanced interaction. All peptides show very similar inhibition of trypsin, consistent with this interaction being specific for the multimeric arrangement found in tryptase. Once again, these results are consistent with the additional lysine residue providing an additional site of interaction to a second site in tryptase.

Interestingly, it is also known that the N-terminal residues of LDTI, Lys1 and Lys2, make important interactions with a negatively charged patch on tryptase made up of the side chains from Asp143 and Asp147.[32] This has been elegantly demonstrated by Stubbs et al.[21] by progressive deletion of Lys1 and Lys2 in the LDTI peptide chain. These modifications result in an increase in K_i from 1.2 nM (LDTI) up to 19 nM (complete deletion) with no concomitant effect on the activity towards trypsin or chymotrypsin. We believe that the BBI peptides that have the N-terminal extension including a lysine are acting as direct mimics of the N-terminal region from LDTI, and are picking up similar interactions with Asp143 and Asp147. The similarity extends to the kinetics, where both LDTI and many of the N-terminally extended peptides show a mixture of tight and weak binding sites, as outlined above.

2.3. Combination of N- and C-terminal extension peptides

The results described so far show that both N- and C-terminal positively charged extensions are able to enhance the inhibitory capability of the starting BBI–peptide sequence. The results are consistent with the terminal lysines being able to bind into a nearby site, possibly an adjacent active site, on the tryptase. However, it was not known whether the different extensions were picking up interactions with the same site or with separate ones. Accordingly, we combined the best N- and C-spacers into one single sequence, resulting in peptides **31**–**33**. The inhibition constants for these peptides are given in Table 5. In each of these combination peptides, the inhibition kinetics are consistent with a single class of binding site and no biphasic inhibition curves are observed. Surprisingly, we find that these peptides

Table 5. Inhibition of β-tryptase and trypsin by BBI-derived peptides: combination of N- and C-terminal extensions

	Sequence	K_i tryptase (μM)	K_i trypsin (μM)
31	KεAhxGGSCTKSIPPQCYγAbuKG	0.0087	0.017
32	KεAhxGGSCTKSIPPQCYβAlaKG-NH_2	0.0029	0.015
33	KεAhxGGSCTKSIPPQCYγAbuKG-NH_2	0.0010	0.024

All peptides incorporate a disulfide bridge linking the two cysteines. Errors in K_i are typically ±10%.

Table 4. Inhibition of β-tryptase and trypsin by BBI-derived peptides: effect of N-terminal charge

	Sequence	K_i tryptase (μM)	K_i(2) tryptase (μM) [%]	K_i trypsin (μM)
22	KεAhxεAhxSCTKSIPPQCY	0.29	43.2 [9.4%]	0.039
27	AcKεAhxεAhxSCTKSIPPQCY	0.31	199 [27.5%]	0.017
28	NleεAhxεAhxSCTKSIPPQCY	10.3	—[a]	0.017
23	KεAhxGGSCTKSIPPQCY	0.076	74 [6.0%]	0.022
29	AcKεAhxGGSCTKSIPPQCY	0.15	185 [14.0%]	0.016
30	NleεAhxGGSCTKSIPPQCY	0.64	91 [60.7]	0.025

Where indicated, the inhibition behaviour of the peptides with tryptase shows bimodal inhibition with the data being characterised by a tight and a weak binding interaction. For the biphasic data, a second K_i value is shown together with an indication of the proportion of the data that are represented by the weaker binding. All peptides incorporate a disulfide bridge linking the two cysteines. Errors in K_i are typically ±10%.

[a] Data are consistent with a single class of binding site only.

show a K_i value that is much improved on either of the peptide series of Tables 1 and 3. The increased potency of these inhibitors resulted in the inhibition curves being characterised by tight-binding kinetics. Again it is found that a C-terminal amide is preferred to a C-terminal acid (compare peptides **31** and **33**). The peptide with the lowest K_i of those tested is **33**, which has a K_i value of 1.0 nM. This value is a 69-fold improvement on the best N-spacer series peptide and 48-fold improvement on the best C-spacer peptide. These results suggest that the N- and C-terminal extensions are providing interactions with distinct sites on the tryptase.

It is worth noting that the K_i value obtained for peptide **33** is lower than that recorded for LDTI (1.4 nM,[3]) and for cyclotheonamides E4 and E5 (K_i = 5.1 and 84.7 nM, respectively[23]), which are the only natural peptidic human tryptase inhibitors known so far. In addition, unlike LDTI, peptide **33** does not display a mixture of tight and weak inhibition sites, resulting in considerably improved inhibition properties.

The starting BBI peptide (**1**) is a far more potent inhibitor of bovine trypsin than it is of human tryptase (Table 1). In general, none of the modifications that are described in this paper have much effect on the activity against trypsin. This is because they are designed to pick up second-site interactions that are only present in the multimeric tryptase. By incorporating both N- and C-terminal extensions to the BBI peptide it has proved possible to improve the potency against tryptase by over four orders of magnitude, with the most potent inhibitor (**33**) having a 24-fold lower K_i for tryptase than it has for trypsin.

3. Conclusions

In this paper, we report a series of peptides based on the sequence SCTKSIPPQCY (**1**), which is derived from the solvent exposed loop of the Bowman–Birk Inhibitor. The modifications, which comprise a peptide chain added on either the C- or the N-terminus of the peptides, aimed to realise multi-headed inhibitors for human lung β-tryptase.

The addition of an N-terminal lysine, incorporating a suitable spacer, was found to improve potency towards tryptase. Although it was not our original intention, the optimal inhibitors show many characteristics of LDTI—the separation of the terminal lysine from the P_1 is at a comparable distance to that of the N-terminal Lys-Lys in LDTI and the inhibition kinetics become multiphasic. We therefore suggest that these N-terminally extended BBI peptides can be considered LDTI analogues.

The peptides constructed with C-terminal extensions also show improved inhibition properties against tryptase. In this case, however, the inhibition kinetics remain simple. We believe that the improved inhibitory properties are due to interactions with an adjacent active site and that these C-terminally extended peptides are functionally similar to the two-headed inhibitors that have been described by other authors.[9,13,14,16,18,26,31]

Combination of N- and C-terminal extensions is found to produce a peptide that has a synergistic improvement in potency. The best sequence (**33**) has a K_i value (1.0 nM) that is improved by a factor greater than 10^4 over the starting BBI peptide sequence (**1**, K_i = 31.5 μM). The synergy suggests that these extensions are picking up interactions with distinct regions of tryptase. We therefore consider that peptide **33** is acting as a tri-functional inhibitor. Remarkably, this inhibitor is more potent that the best natural protein inhibitor (LDTI) as well as displaying more favourable inhibition kinetics.

4. Experimental

4.1. Peptide synthesis

Solid phase peptide synthesis was performed on a Shimadzu PSSM-8 multiple synthesiser, using Fmoc chemistry and the first amino acid immobilised on Wang resin. Disulfide bond formation between the Cys residues was obtained as described previously.[33] Purification of peptides was by preparative HPLC using a Gilson 712 system equipped with a Waters Nova-Pak C_{18} 6 μm 100 × 25 mm, reverse-phase column, using a water–acetonitrile gradient eluant buffered with 0.1% TFA. Signals were monitored at 223 nm with an ABI759A UV-detector. All peptides were characterised by FAB^+ and analytical HPLC equipped with Vydac C_{18} 5 μm 150 × 4.6 mm, reverse-phase column. All *N*-Fmoc-amino acids and Wang-*N*-Fmoc-amino acids resins were purchased from NovaBiochem Ltd (UK) with the following side chain protecting groups: Cys (Trt), Gln (Trt), Lys (Boc), Ser (tBu), Thr (tBu), Tyr (tBu).

4.2. Biological assays

Human rhLung β tryptase was purchased from Promega Ltd (UK). Trypsin from bovine pancreas was purchased from Sigma. Low molecular weight heparin from porcine intestinal mucosa was purchased from ICN Pharmaceuticals Ltd (UK). Chromogenic substrates Ts-Gly-Pro-Lys-pNA[34] and Bz-L-Arg-AMC,[35] used for tryptase and trypsin, respectively, were purchased from Sigma.

4.2.1. Tryptase assay. Biological assays were performed at pH 7.8 using a 50 mM Tris–HCl and 150 mM NaCl buffer containing 110 μg mL^{-1} of heparin. Peptides were prepared as 1 mg mL^{-1} stock solutions (maximum concentration in assay 100 μM). Inhibition assay was carried out in a total volume of 200 μL of buffer wherein β-tryptase (50 μL, final concentration 1 nM) was incubated for 5 min with various concentrations of peptides (100 μL). The reaction was started by the addition of substrate Ts-Gly-Pro-Lys-pNA (50 μL, final concentration 79 μM) and, after 1 min standing and 1 min shaking, the residual activity was measured on a microplate reader (Ceres UV900HDi, Bio-Tek Instruments Inc.) at room temperature in the kinetic mode over 20 min at

405 nm. The enzyme concentration was determined using the formula $[E]_0 = v\cdot(K_m + [S])/(k_{cat}\cdot[S])$, where v is the reaction velocity.

4.2.2. Trypsin assay. Biological assays were performed at pH 8.0 using a 50 mM Tris–HCl and 20 mM $CaCl_2$. Peptides were prepared as 1 mg mL^{-1} stock solutions (maximum concentration in assay 2 μM). Inhibition assay was carried out in a total volume of 200 μL of buffer wherein trypsin (50 μL, final concentration 5 nM) was added to various concentrations of peptide (100 μL) immediately followed by the addition of substrate Bz-L-Arg-AMC (50 μL, final concentration 55 μM). The hydrolysis of the substrate was followed on a microplate reader (CytoFluor Series 4000, Perseptive Biosystems) at room temperature for 10 min at 360 ± 40 nm (excitation) and 460 ± 40 nm (emission).

4.3. Inhibition calculations

K_i values for each enzyme were obtained by fitting the initial velocity of substrate hydrolysis to the appropriate equation by nonlinear regression using the GraFit software programme.[36] Where the data were consistent with a single class of binding site, they were fitted using Eq. 1, where v_0 is the uninhibited reaction rate and $K_{i(app)}$ is the apparent K_i value.

$$v = v_0\left(1 - \frac{[I]}{K_{i(app)}}\right), \tag{1}$$

where the inhibition showed more than a single class of site, the data were fitted to a two site equation (Eq. 2)

$$v = v_{0(1)}\left(1 - \frac{[I]}{K_{i1(app)}}\right) + v_{0(2)}\left(1 - \frac{[I]}{K_{i2(app)}}\right). \tag{2}$$

In Eq. 2, uninhibited reaction rate is considered the sum of the rates, $v_{0(1)}$ and $v_{0(2)}$ from the two sites; the two apparent K_i values are $K_{i1(app)}$ and $K_{i2(app)}$.

For the most potent inhibitors it is necessary to fit data to a tight binding equation (Eq. 3)

$$v = v_0 - \frac{v_0([E]_0 + [I] + K_{i(app)} - \sqrt{([E]_0 + [I] + K_{i(app)})^2 - 4[E][I]})}{2[E]_0}. \tag{3}$$

K_i values were determined from $K_{i(app)}$ by correction for substrate competition using Eq. 4

$$K_i = \frac{K_{i(app)}}{1 + \frac{[S]}{K_m}}. \tag{4}$$

4.3.1. Molecular modelling. To give an indication of the (maximum) separation between the lysine residue at P_1 and the lysine residue positioned at the N- or C-terminus of the peptide chains, the various peptide extensions were modelled on to the respective ends of the BBI loop co-ordinates from the NMR minimised mean structure.[37] The extensions were built in their fully extended conformations, and the distance measured between the carbonyl carbon atoms of the two lysine residues.

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