Enzyme Inhibition

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Reversible and Noncompetitive Inhibition of β-Tryptase by Protein Surface Binding of Tetravalent Peptide Ligands Identified from a Combinatorial Split–Mix Library**

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Protein surface recognition has been become a promising tool in recent times not only to study biomolecular recognition events, but also to control or modulate the function of the target protein. Besides the small molecules identified from high-throughput screening of large and random compound libraries, rationally designed protein surface binders have also been developed, most often based on protein secondary structure mimetics.[1,2] Binding to the protein is thought to involve a combination of electrostatic and hydrophobic interactions, as most proteins present a distinct and unique pattern of hydrophobic and charged residues on their surface.[3] Polymers and multivalent peptide ligands have also been shown to interact with proteins.[4] Protein surface binders often modulate protein function by disrupting protein–protein interactions or inducing denaturation of the target protein.

In the last few years, we have studied the stereoselective recognition of small peptide fragments in water by artificial receptors identified from combinatorial split–mix libraries.[5] Therefore, we now wanted to extend this approach to proteins. We chose the serine protease β-tryptase, which plays an important role in the pathogenesis of asthma and other allergic and inflammatory disorders, as a target.[6] The structure of tryptase consists of a framelike tetramer with four active sites directed towards a central pore.[7] The tetramer is held together by heparin, a negatively charged polysaccharide. In the absence of heparin, tryptase irreversibly dissociates into inactive monomers.[8] A large variety of inhibitors for β-tryptase are known. Dibasic inhibitors,[9] which bind to two neighboring of the four active sites simultaneously, or cyclotides (macrocyclic cysteine knotted miniproteins with 26–37 amino acids)[10] are reversible competitive inhibitors, whereas heparin antagonists, such as lactoferrin, a 78 kDa protein, or the small arginine-rich protein protamine, induce the irreversible denaturation of the enzyme.[11]

We wanted to explore whether β-tryptase can also be inhibited by small and flexible molecular receptors that bind to its surface. Even though the enzyme has a pI value of about 5.0 to 6.6,[12] which indicates only a slightly negatively charged surface, there are some highly acidic "hot spots" with clusters of negatively charged amino acids (Glu, Asp).[7] These areas are mainly found at the entrance to the central pore, which allows access to the active cleavage sites buried within this cavity (Figure 1). A multivalent cationic peptide ligand should therefore be able to interact with these surface hot spots, thereby not only tightly binding to the enzyme's surface but at the same time also blocking the entrance to the active sites, thus leading to enzyme inhibition.

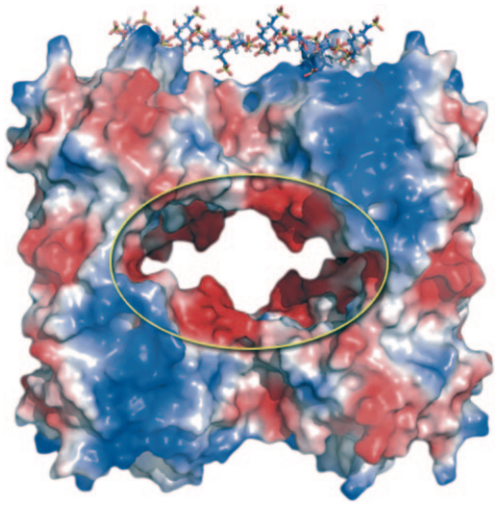

Figure 1. The surface of β-tryptase features clusters of negatively charged amino acids (red) around the entrance to the central cavity in which the active sites are located. Heparin (schematic structure on top of the enzyme) stabilizes the tetrameric structure of β-tryptase.

For the design of our inhibitors we chose a second-generation lysine dendrimer to which four identical tetrapeptide arms[13] were attached (Figure 2). For the synthesis of the lysine trimer scaffold as well as the peptide arms, standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis was applied. Each arm contained three variable positions AA^1–AA^3. To identify the optimum sequence, a combinatorial approach was chosen with the following six proteinogenic L-amino acids at each variable position: lysine, arginine, tryptophan, glutamic acid, phenylalanine, and alanine. These amino acids were selected because they

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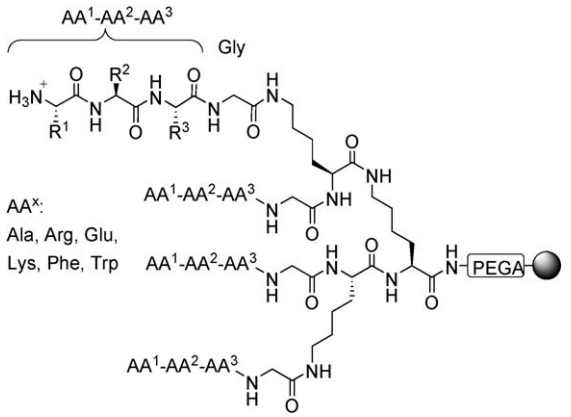

Figure 2. Combinatorial library of 216 tetravalent inhibitors with four identical arms, in which the last three amino acids are combinatorially varied with six amino acids in each variable position. PEGA = polyethylene glycol–polyacrylamide copolymer.

cover a wide range of different physicochemical properties, and have basic (lysine, arginine), acidic (glutamate), aromatic (tryptophan, phenylalanine), and aliphatic (alanine) side chains. We have shown before that the use of such a small, but focused, combinatorial library can be as efficient as the use of a large, but random, library with respect to the screening results.[14]

Hence, a combinatorial split–mix library with $6^3 = 216$ different tetravalent peptide ligands was synthesized on biocompatible PEGA resin[15] by using a "split and mix" approach and the IRORI radio-frequency tagging technology,[16] which provides each library member in macroscopic quantities spatially separated in a small microreactor. Details of the synthesis can be found in the Supporting Information.

After synthesis the library was screened directly on-bead for inhibition of tryptase in a high-throughput fluorescence assay by means of a microplate reader with 96-well plates and Tos-Gly-Pro-Arg-AMC as substrate, which is cleaved into the highly fluorescent 7-amino-4-methylcoumarin (AMC). The direct on-bead screening allows a quantitative readout of the inhibition data of all library members. In contrast to inhibitors in solution, solid-phase-bound compounds require significantly longer incubation times in an on-bead enzyme assay (hyperentropic efficacy[17]). Therefore, the enzyme (heparin-stabilized rhSkin β-tryptase, rh = recombinant human) was incubated with the library members for 20 h at room temperature before its remaining activity was determined. Under these conditions the various library members inhibit the enzyme from 95% for the best to < 10% for the weakest inhibitors. Table 1 shows the ten best inhibitors, all with an inhibition above 90%, the best ones being $(RWKG)_4$ and $(KWKG)_4$ with approximately 95% inhibition each.

A closer inspection of the quantitative screening results of the 216 library members showed that lysine and arginine, the two basic amino acids in the library, are the most important amino acids for obtaining strong inhibition (Figure 3). On the contrary, glutamate is almost not present in the best inhibitors but occurs most often in the weak or inactive peptide ligands. The most important position seems to be AA^3. The best

Table 1: The ten best inhibitors of the on-bead screening (inhibition in %; cationic amino acids are marked in bold).

AA^1	AA^2	AA^3	Inhibition[a]
Arg	Trp	**Lys**	95
Lys	Trp	**Lys**	94
Trp	**Lys**	Phe	93
Phe	**Arg**	**Lys**	92
Lys	**Arg**	**Arg**	91
Phe	**Lys**	**Arg**	91
Lys	Trp	**Arg**	90
Lys	Phe	**Arg**	90
Lys	**Lys**	Trp	90
Phe	Trp	**Lys**	90

[a] See the Supporting Information for details of the on-bead assay.

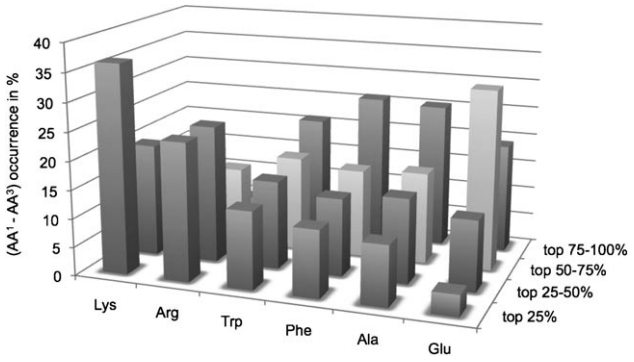

Figure 3. Combined occurrence of the six amino acids used within the library over all three variable positions AA^1–AA^3 ranked according to the inhibition properties of the corresponding ligands (*y* axis: occurrence in %, *z* axis: all inhibitors were divided into four subgroups according to their activity). The cationic amino acids lysine and arginine dominate within the best inhibitor group (top 25%).

inhibitors almost always have a cationic amino acid in this position, whereas glutamate at this position leads to a significant loss of activity even if two cationic amino acids follow in positions AA^2 and AA^1 (e.g., only 58% inhibition for $(RREG)_4$ and 48% for $(KKEG)_4$). In the case of the best inhibitor $(RWKG)_4$, the activity is reduced by almost half when lysine at position AA^3 is replaced with glutamate. ($(RWKG)_4 = 95\%$ and $(RWEG)_4 = 48\%$, respectively).

Interestingly, the inhibition is particularly high when basic amino acids are combined with aromatic amino acids. Seven out of the ten best inhibitors (Table 1) contain two basic amino acids together with either phenylalanine (three times) or tryptophan (four times). Hence, this combination seems to provide the strongest interaction with tryptase.[1,4] In particular, the combination of tryptophan in position AA^2 surrounded by basic amino acids seems to be very efficient. Perhaps unexpectedly in contrast to this, inhibitors with three basic amino acids are only moderately active: $(KKKG)_4$ is ranked in 39th place within the library and $(RRRG)_4$ in only 52nd place.

To characterize the inhibition properties of these tetravalent peptide ligands in more detail and to further elucidate their mode of action, the best inhibitor $(RWKG)_4$ as well as the slightly less active $(KKFG)_4$, along with their monovalent

analogues, the two linear tetrapeptides H-RWKG-NH_2 and H-KKFG-NH_2, were resynthesized in larger amounts by using a standard solid-phase peptide synthesis protocol. The inhibition properties of these four compounds were determined in a standard solution-phase fluorescence assay. Inhibition constant (K_i) values were determined by varying the concentration of the substrate from 1000 to 0.01 μM.[18]

These solution-phase inhibition studies confirmed the results from the on-bead screening (Table 2). Peptide ligand $(RWKG)_4$ with a K_i value of 170 nM is a highly efficient inhibitor of β-tryptase, whereas $(KKFG)_4$ is a little less active

Table 2: Enzyme selectivity: K_i values [μM] for the four compounds tested in solution (plus the known literature inhibitor *p*-Ab[18] as a standard) for three different enzymes.

Compound	β-Tryptase	Trypsin	Chymotrypsin
p-Ab	56.97 ± 8.25	5.29 ± 0.80	> 1000
KKFG	471.74 ± 31.27	> 1000	> 1000
$(KKFG)_4$	0.63 ± 0.02	> 1000	> 1000
RWKG	305.83 ± 50.22	> 1000	> 1000
$(RWKG)_4$	0.17 ± 0.02	> 1000	> 1000

(K_i value: 630 nM), in agreement with its lower activity in the on-bead screening (95% for $(RWKG)_4$ compared to 84% for $(KKFG)_4$). However, a comparison of the tetravalent peptide ligands with their monovalent analogues revealed a significant multivalency effect. $(KKFG)_4$ is more active by a factor of 750 than H-KKFG-NH_2 (630 nM versus 472 μM), whereas the best inhibitor $(RWKG)_4$ is 1800 times more active than H-RWKG-NH_2, its monovalent counterpart (170 nM versus 306 μM).

Enzyme selectivity was tested against trypsin and chymotrypsin, two related serine proteases that are structurally very similar to the corresponding tryptase monomer. Furthermore, the active site of trypsin is very similar to the active site of β-tryptase. In particular, the S1 specificity pocket is nearly identical in both enzymes.[19] However, as the data in Table 2 show, all four compounds are inactive against either trypsin or chymotrypsin, at least under the conditions studied ($K_i >$ 1000 μM).[20]

Further studies showed that inhibition of β-tryptase by the peptide ligands examined is reversible (enzyme activity could be restored by dialysis with buffer). Another variant of tryptase, heparin-free rhLung β-tryptase,[21] is also inhibited by these ligands (e.g., $(KKFG)_4$ has a K_i value of 323 nM). Hence, heparin (a polyanion and a possible binding partner for cationic molecules) cannot be the target of our inhibitors. Therefore, the peptide ligands must bind directly to the protein part of β-tryptase.

To determine whether the inhibitors interact with the active site of the enzyme or with the protein surface, the inhibition profile was measured at different inhibitor concentrations ([I] = 0, 1, 10, and 100 μM). Analysis of the kinetic data clearly shows that our tetravalent peptide ligands are noncompetitive inhibitors. With increasing inhibitor concentration, the maximum reaction rate v_{max} for the enzymatic reaction significantly decreases, but in all cases $v_{max}/2$ is reached at similar substrate concentrations [S], thus indicating a similar Michaelis constant (K_m) value (Figure 4).

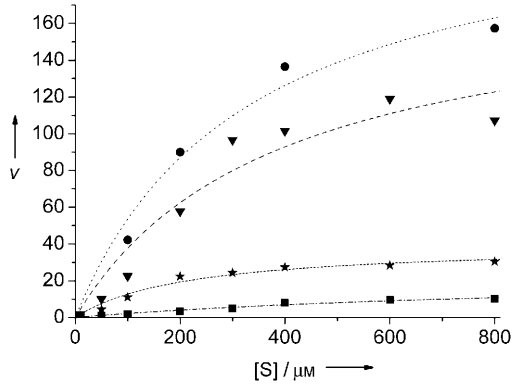

Figure 4. Test for competitive versus noncompetitive inhibition (shown for tetravalent ligand $(KKFG)_4$). With increasing inhibitor concentration [I], the maximum reaction rate (v_{max}) decreases, which indicates noncompetitive inhibition ([I] = 0 (●), 1 (▼), 10 (⋆), and 100 (■) μM).

Hence, the tetravalent peptide ligands are not in direct competition with the enzyme's substrate. They are reversible, noncompetitive inhibitors and thus do not bind to the active site of the enzyme (in contrast to all other previously reported reversible inhibitors for tryptase). Interestingly, the two linear peptides (the monovalent analogues of the tetravalent peptide ligands) are classical but weak competitive active-site inhibitors (data not shown). Therefore, not only is a dramatic increase in affinity observed when going from a monovalent to a tetravalent peptide ligand, but also a switch in inhibition mechanism (from competitive to noncompetitive). Hence, our tetravalent peptide ligands are, to the best of our knowledge, the first reversible and simultaneously noncompetitive inhibitors for β-tryptase reported so far.

In conclusion, we have shown that the quantitative on-bead enzyme screening of a combinatorial split–mix library of flexible tetravalent peptide ligands reveals highly efficient inhibitors with nanomolar affinity and remarkable enzyme selectivity. They are the first reported noncompetitive but reversible inhibitors for β-tryptase. All experimental data are in agreement with an unusual inhibition mode: the peptide ligand most likely binds to the surface of the protein and, like a "molecular plug", prevents access of the substrate to the active sites. We are currently exploring whether tetravalent peptide ligands with different side chains or a rigid branching element are even more advantageous.

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