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Introduction of non-natural amino acid residues into the substrate-specific P_1 position of trypsin inhibitor SFTI-1 yields potent chymotrypsin and cathepsin G inhibitors

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ABSTRACT

A series of trypsin inhibitor SFTI-1compounds modified in substrate-specific P_1 position was synthesized by the solid-phase method. Lys5 present in the wild inhibitor was replaced by Phe derivatives substituted in *para* position of the phenyl ring, L-pyridylalanine and *N*-4-nitrobenzylglycine. Their inhibitory activities with bovine α-chymotrypsin and cathepsin G were estimated by determination of association equilibrium constants (K_a). All analogues inhibited bovine α-chymotrypsin. The highest inihbitory activity displayed peptides with the fluorine, nitro and methyl substituents. They were 13–15-fold more active than [Phe^5]SFTI-1 used as a reference. They are the most potent chymotrypsin inhibitors of this size. Substitution of Lys5 by Phe did not change the cathepsin G inhibitory activity. Introduction of Phe(*p*-F), Phe(*p*-NH_2) and Phe(*p*-CH_3) in this position retained the affinity towards this proteinase, whereas Phe(*p*-guanidine) gave an inhibitor more than twice as active, which appeared to be stable in human serum. On the other hand, a peptomeric analogue with *N*-4-nitrobenzylglycine failed to inhibit cathepsin G. Despite the fact the introduced amino acids were non-coded, the peptide bonds formed by them were hydrolyzed by chymotrypsin. We postulate that additional interaction of *para*-substitutents with the enzyme are responsible for the enhanced inhibitory activity of the analogues.

1. Introduction

Almost one-third of all proteases have been classified as serine proteinase.[1] They are responsible for a broad spectrum of biological activity. Uncontrolled activity of these enzymes can be very dangerous for the organism. Therefore a very strict mechanism of their control (in both time and site) is required. One of the predominant mechanisms rests on ubiquitous presence of inhibitors of these proteinases. It turned out that many, but not all serine proteinase inhibitors, display the same so-called standard mechanism of association with their target enzymes.[2] They interact with enzymes in a substrate-like fashion through an exposed fragment named binding loop which is characterized by unique canonical conformation.[3] **A** hyper exposed amino acid residue located in the centre of this loop, named after Schechter and Berger[4] P_1 residue, interacts with S_1 cavity of the enzyme accounting for up to 50% of the inhibitor–enzyme contacts.[5,6] P_1 residue determines the inhibitor specificity. In many cases, a single substitution of an amino acid residue in this position in naturally occurring serine proteinase inhibitor changes dramatically their specificity as shown by systematic studies on analogues modified in P_1 position of turkey ovomucoid third domain[5] and bovine pancreatic trypsin inhibitor.[6] Since both inhibitors are proteins, the appropriate changes were mainly introduced using site-directed mutagenesis (few non-coded amino acids were inserted into the amino acid sequence by enzymatic semisynthesis[5]) and therefore focused on coded amino acids. Extensive studies on the influence of amino acid residue present in the position on inhibitor specificity were also performed on shorter compounds like peptides based on the binding loop of Bowman-Birk inhibitor.[7] But also in this case, mainly proteinogenic amino acids were investigated. One of the main goals behind such investigations is to develop a drug candidate to treat several diseases including arthritis, rheumatoid arthritis, hemophilia, emphysema, neurodegenerative disorders, inflammatory and allergic disorders (including asthma) and many others. For several reasons, including poor bioavailability and susceptibility to degradation by proteinases, application of proteins and peptides as drugs is rather limited. One of the obvious approaches to overcome these obstacles is introduction of non-coded amino acids into the peptide chain. In our previous works we have shown that despite a very strict structural requirements for inhibitor–enzyme P_1–P_1' interactions,[8] some of the artificial amino acid residues are well tolerated.

In past years, we focused our attention on trypsin inhibitor SFTI-1 isolated from sunflower seeds.[9] This dicyclic 14-amino acid

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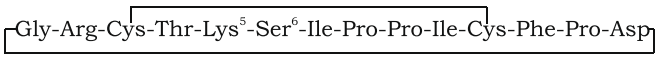


Figure 1. Primary structure of trypsin inhibitor SFTI-1.

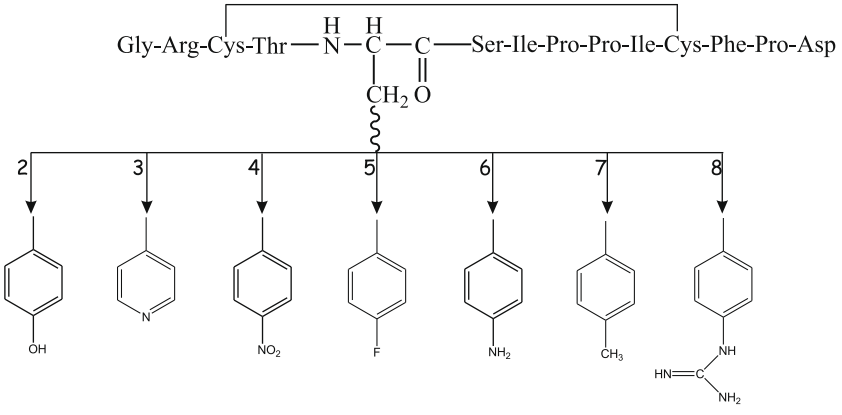
residues inhibitor (Fig. 1) contains Lys5 in P_1 position and therefore exhibits affinity towards trypsin-like proteinases. By the synthesis of active SFTI-1 analogues modified in position P_1 with hydrophobic α-hydroxymethylamino acids, we have demonstrated[10] that the ion pair formation between the enzymic β-carboxyl group of Asp189 and the positively charged side chain group of an amino acid residue present in P_1 position of the inhibitor, the main source of the enzyme–inhibitor interaction can be successfully replaced by a network of hydrogen bonds. Recently, we have synthesized SFTI-1 analogues which contained in this position *N*-(4-aminobutyl)-glycine and *N*-benzylglycine that mimic Lys and Phe, to obtain trypsin and chymotrypsin inhibitors, respectively.[11]

It is well documented that aromatic residues in inhibitor P_1 are optimal for interactions with chymotrypsin. Enhanced activity of chymotrypsin inhibitors having Tyr in position P_1, as compared to those with Phe[5,6] suggests that additional *p*-hydroxyl group stabilizes the chymotrypsin-inhibitor complex. Unfortunately, there are no X-ray data available supporting this statement. Taking into consideration these results, we decided to synthesize and determine inhibitory activity of a series of SFTI-1 monocyclic analogues (with disulfide bridge only) modified in substrate-specific P_1 position by Phe residues substituted in the benzene ring and, in addition, one peptomeric analogue with *N*-4-nitrobenzylglycine (Nphe(*p*-NO_2). Our purpose was to determine the influence of the *para*-substituent on enzyme–inhibitor interaction. Chemical formulas of the Phe derivatives are shown in Figure 2.

The other enzyme which was the subject of our investigations was cathepsin G. It combines both chymotrypsin-like and trypsin-like specificities which is not very common among proteinases. Duodenase and a crab fiddler collagenase are two examples of such enzymes.[12,13] Glu226 of cathepsin G divides the bottom of substrate pocket into sub-compartments that can accommodate positively charged side chains of Lys or Arg or phenyl ring of Phe or Tyr. As shown in Figure 3, some of the Phe derivatives [Phe(*p*-NH_2), Phe(*p*-guanidine)] introduced into the position of the SFTI-1 analogues display both aromatic and basic character and therefore they are likely to facilitate the interaction of these analogues with cathepsin G.

2. Results and discussion

The determined molecular weights and the results of HPLC analysis of the synthesized peptides, together with their inhibitory activities, are summarized in Table 1. All the synthesized SFTI-1 analogues are monocyclic with free C-terminal carboxyl group. Conversion of this group to amide reduced their inhibitory activity. In our previous work[15] we have shown that one of the cyclic elements can be removed from SFTI-1 without significant loss of the trypsin inhibitory activity. Replacement of Lys5 only, present in inhibitory P_1 position, by Phe in monocyclic SFTI-1 with a disulfide bridge, increased 400-times the inhibitory activity against bovine α-chymotrypsin, reaching an association equilibrium constant as high as $2.0 \times 10^9\ M^{-1}$. [Phe^5]SFTI-1 will be used as a reference compound. All the synthesized SFTI-1 analogues exhibited affinity towards bovine α-chymotrypsin. Substitution of Phe by Tyr yielded about a 10-fold more potent chymotrypsin inhibitor. This is in good agreement with the results obtained by other teams working on proteinous inhibitors: bovine pancreatic trypsin inhibitor (BPTI)[6] and turkey ovomucoid third domain.[5] Nevertheless, in both cases the impact of the additional OH group on inhibitory activity was less pronounced. Among the synthesized analogues, the highest chymotrypsin inhibitory activity, 15 times as high as that of the reference compound, was determined for the one with Phe(*p*-F). Almost equipotent activity was displayed by peptides with uncharged substituents introduced into *para* position of the phenyl ring, [Phe(*p*-NO_2)^5]SFTI-1 and [Phe(*p*-CH_3)^5]SFTI-1. Introduction in this position of a positively charged guanidine group dramatically reduced the affinity towards the experimental enzyme. Interestingly, amino acid residues Pal and Phe(*p*-NH_2) with less basic aromatic amines on their side chains produced potent chymotrypsin inhibitors. It is also worth noting that high chymotrypsin inhibitory activity was determined for peptomeric analogue [Nphe(*p*-NO_2)^5]SFTI-1. Also in this case, the nitro substituent increased



2. L-tyrosine (Tyr)
3. 3-(4'-pyridyl)-L-alanine (Pal)
4. 4-nitro-L-phenylalanine (Phe(p-NO_2))
5. 4-fluoro-L-phenylalanine (Phe(p-F))
6. 4-amino-L-phenylalanine (Phe(p-NH_2))
7. 4-methyl-L-phenylalanine (Phe(p-CH_3))
8. 4-guanidyl-L-phenylalanine (Phe(p-guanidine))

Figure 2. Chemical formulas and abbreviations of Phe derivatives introduced into P_1 position of monocyclic trypsin inhibitor SFTI-1.

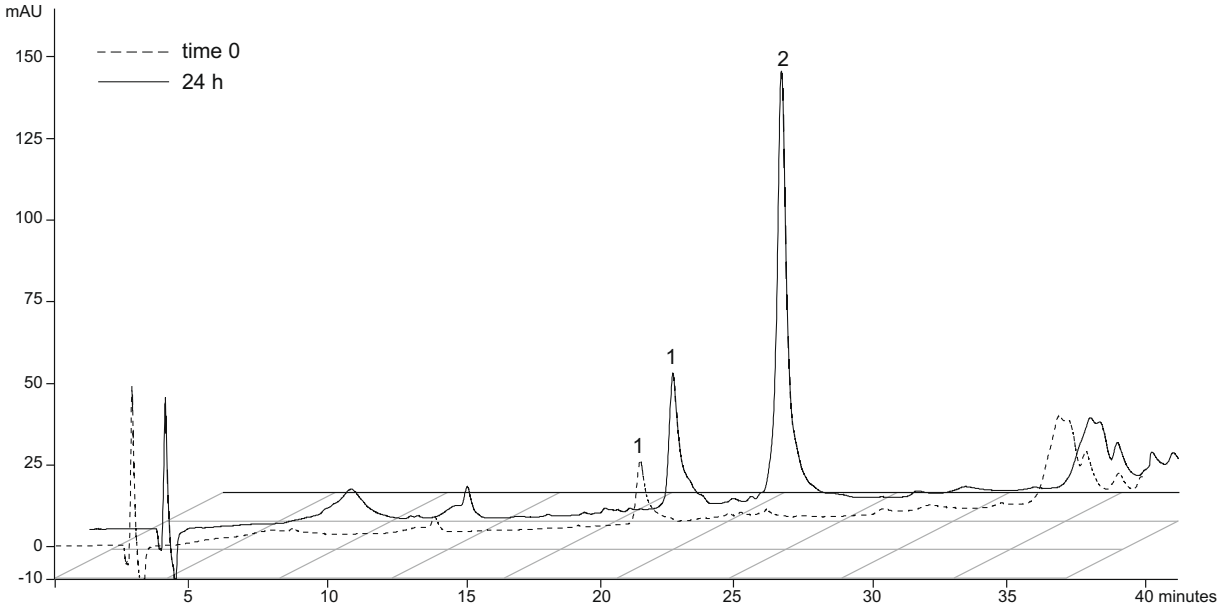


Figure 3. Proteolytic susceptibility of analogue **4** (Gly-Arg-Cys(&)-Thr-Phe(*p*-NO_2)-Ser-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp) (nomenclature of cyclic peptides as recommended by Spengler et al.[18]) in the presence of α-chymotrypsin, at pH 8.3: peak 1—intact peptide, peak 2—peptide with hydrolyzed Phe(*p*-NO_2)—Ser peptide bond. Peaks with retention times above 30 min come from Triton X-100.

inhibitory activity by almost one order of magnitude. The K_a values determined for analogues **4**, **5** and **7** with chymotrypsin indicate that they are the most potent chymotrypsin inhibitors of this size reported to date.

It is well documented that in canonical inhibitors, the peptide bond formed by P_1 and P'_1, called the reactive site, is selectively cleaved by a cognate enzyme. In neutral pH, the hydrolysis constant between the intact and hydrolyzed inhibitor is close to unity.[16] Unlike substrates, standard-mechanism inhibitors are characterized by slow hydrolysis of the P_1–P'_1 reactive site. It is reasonable to assume that introduction of non-coded amino acids in P_1 position should increase proteolytic stability of the reactive site of the inhibitor and might increase inhibitory activity. In our recent work,[17] we have shown that introduction of the Nphe residue in P_1 position decreased the hydrolysis rate of the reactive site of inhibitors based on the SFTI-1 structure. Hydrolysis patterns for the three compounds: two most potent inhibitors [Phe(p-NO_2)^5]SFTI-1 (**4**) (Fig. 3), [Phe(*p*-F)^5]SFTI-1 (**5**) and the reference compound [Phe^5]SFTI-1 (not shown) were similar. In all cases the peptide bond between amino acid residues in position P_1 and P'_1 underwent gradual hydrolysis until the equilibrium between the intact and hydrolyzed forms of the inhibitor (peaks 1 and 2, respectively) was reached. No further progress in the proteolysis was detected, which indicates that the peptides belong to the classical canonical group of inhibitors, where the equilibrium between the intact and hydrolyzed form of the peptide is observed. This phenomenon is not observed when the amino acid residue in position P_1 of the inhibitor is replaced by a peptoid residue. Substitution of Phe(*p*-NO_2) by Nphe(*p*-NO_2) in this position of the peptide results in a proteolytically resistant analogue (see Fig. 4).

Surprisingly enough, our research has shown that native SFTI-1 does not have such high activity against cathepsin G as that claimed by Luckett et al. ($K_i \sim 0.15$ nM).[9] When we calculated the K_i using our experimental data collected for the determination of K_a, we obtained a value of 570 nM which is approximately three orders of magnitude higher. Unfortunately, the cited report does not contain any details about the measurement of K_i value. Thus, it is impossible to find the origins of these discrepancies. Since

Table 1
Physicochemical properties and association equilibrium constants (K_a) of SFTI-1 analogues with bovine α-chymotrypsin and cathepsin G

Inhibitor[a]	MW calcd (found)	HPLC[b] rt (min)	Bovine α-chymotrypsin		Cathepsin G	
			K_a (M^{-1})	Relative activity	K_a (M^{-1})	Relative activity
SFTI-1 (dicyclic)			$(5.2 \pm 1.5) \times 10^6$ [14]		$(5.7 \pm 0.3) \times 10^6$	1.54
SFTI-1			$(5.0 \pm 1.4) \times 10^6$ [14]		n.d.	
[Phe^5]SFTI-1	1550.7 (1550.6)	19.6	2.0×10^9	1.00	$(3.7 \pm 0.23) \times 10^6$	1.00
[Nphe^5] SFTI-1	1550.7 (1550.8)	19.5	$(3.8 \pm 0.3) \times 10^8$ [11]	0.19	N/A	
[Nphe^5] SFTI-1(NH_2)	1549.8 (1549.5)	19.7	$(1.0 \pm 0.1) \times 10^7$	Below 0.01	N/A	
[Nphe(*p*-NO_2)^5]SFTI-1 (**1**)	1595.8 (1595.2)	19.8	$(2.4 \pm 0.4) \times 10^9$	1.20	N/A	
[Tyr^5] SFTI-1 (**2**)	1566.8 (1566.6)	18.9	$(1.9 \pm 0.2) \times 10^{10}$	9.50	Below 10^5	
[Pal^5]SFTI-1 (**3**)	1552.8 (1552.2)	17.0	$(1.1 \pm 0.1) \times 10^{10}$	5.50	Below 10^5	
[Phe(*p*-NO_2)^5]SFTI-1 (**4**)	1595.8 (1595.6)	20.3	$(2.6 \pm 0.3) \times 10^{10}$	13.00	Below 10^5	
[Phe(*p*-F)^5]SFTI-1 (**5**)	1568.8 (1568.4)	20.6	$(3.0 \pm 0.6) \times 10^{10}$	15.00	$(3.8 \pm 0.2) \times 10^6$	1.02
[Phe(*p*-NH_2)^5]SFTI-1 (**6**)	1565.8 (1565.5)	17.7	$(1.9 \pm 0.3) \times 10^{10}$	9.50	$(3.6 \pm 0.2) \times 10^6$	0.97
[Phe(*p*-CH_3)^5]SFTI-1 (**7**)	1564.8 (1564.3)	21.1	$(2.5 \pm 0.3) \times 10^{10}$	12.50	$(2.8 \pm 0.2) \times 10^6$	0.76
[Phe(*p*-guanidine)^5]SFTI-1 (**8**)	1607.9 (1607.5)	18.9	$(1.8 \pm 0.1) \times 10^8$	0.09	$(8.1 \pm 0.4) \times 10^7$	2.19

[a] Except wild SFTI-1, all inhibitors are monocyclic with disulfide bridge only.
[b] Conditions for HPLC analysis are described in Section 3; N/A—not active, n.d.—not determined.

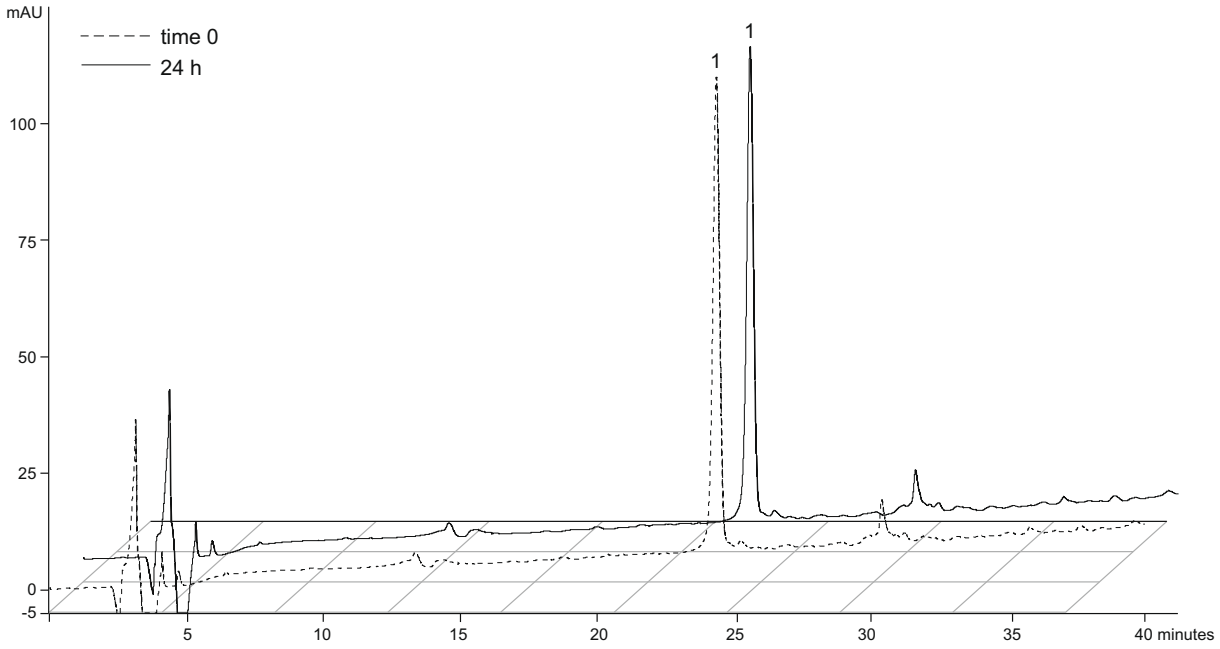


Figure 4. Proteolytic susceptibility of analogue **1** (Gly-Arg-Cys(&)-Thr-Nphe(p-NO_2)-Ser-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp) in the presence of α-chymotrypsin, at pH 8.3.

all our experiments were carried out under the same conditions, we will use our K_a value as a reference in further discussion. As a result of substitution of Lys5 by Phe, the inhibitory activity against this enzyme was retained. This is in line with the substrate specificity of cathepsin G which can accommodate in its S_1 cavity the side chains of both aromatic and positively charged amino acids. As reported by Polanowska et al.[19] cathepsin's G inhibitory activity of BPTI with Lys and Phe present in position P_1 was almost the same and the measured K_a values were of the same order of magnitude as those determined for SFTI-1 and [Phe^5]SFTI-1. Only four of the synthesized SFTI-1 analogues exhibited a considerable inhibitory activity. The most potent, twice as active as the reference inhibitor, appeared to be analogue **8** with p-guanidine-L-phenylalanine in P_1 position. Introduction of fluorine, amino or methyl substituents in *para* position of the phenyl ring did not affect association of the SFTI-1 analogue with cathepsin G. On the other hand, the hydroxyl and nitro groups or introduction of a nitrogen atom into the phenyl ring (analogue **3** with Pal in P_1) gave SFTI-1 analogues with low cathepsin G inhibitory activity. All three analogues with peptoid monomers did not inhibit this proteinase. It is worth emphasizing that the peptoid monomers introduced in inhibitor's P_1 position are well tolerated by trypsin, chymotrypsin and elastase.[11,20]

HPLC analysis of analogue **8** (with Phe(p-guanidine) in P_1 position) incubated with human serum (Fig. 5) reveal that peptide remains stable up to 24 h. It is worth noting that after incubation of

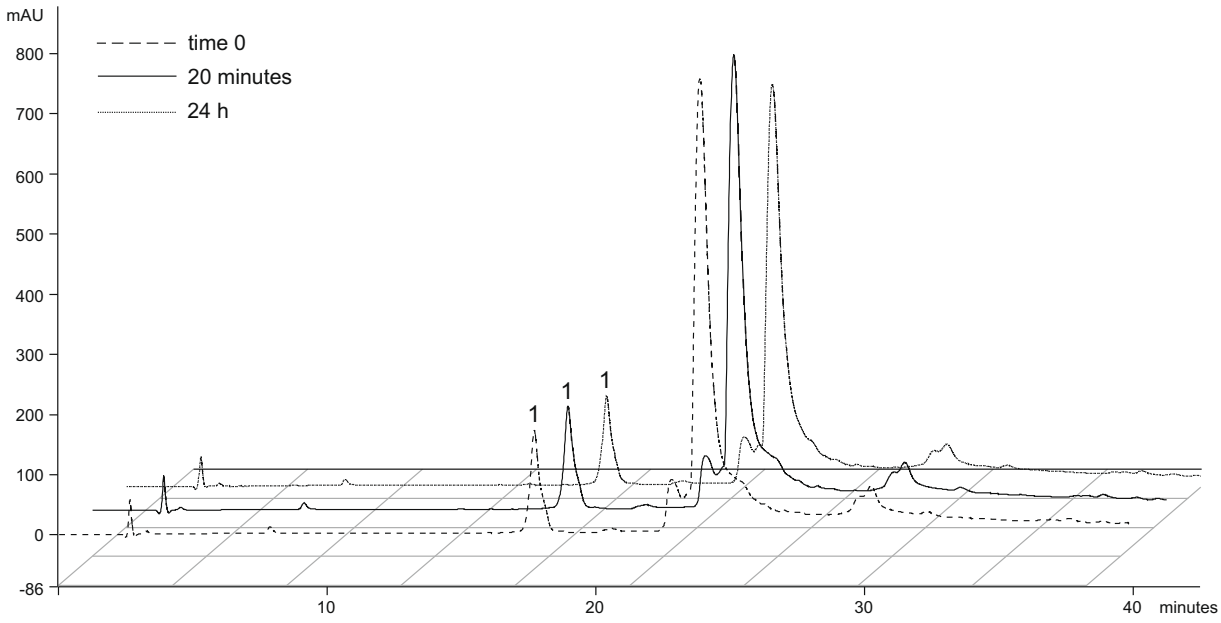


Figure 5. HPLC analysis of analogue **8** (Gly-Arg-Cys(&)-Thr-Phe(p-guanidine)-Ser-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp) after 20 h of incubation with human serum. Peaks with retention times above 18 min come from human serum.

the serum with each substrate, an increasing absorption of ANB-NH_2 was observed indicating the presence of active form of cathepsin G, proteinase 3 and human leukocyte elastase, respectively.

To the best of our knowledge, analogue **8** is one of the most potent inhibitors of cathepsin G that has been reported to date. Taking into account its high serum stability, similar compounds could be candidates for designing a novel class of therapeutic agents.

The results discussed above correlate well with our previous data obtained on chromogenic substrates.[21,22] We synthesized a series of short-chain peptides containing as a *C*-terminal residue the same Phe derivatives with attached 5-amino-2-nitrobenzoic acid amide (ANB-NH_2) to their carboxyl function. In such designed substrates, Phe(X) derivatives were located in P_1 position and ANB-NH_2 was released upon interaction with enzyme. The highest specificity constants (k_{cat}/K_M) with bovine α-chymotrypsin, used as a measure of substrate activity, were obtained for peptides with Phe(*p*-NO_2), Tyr and Phe and Phe(*p*-NH_2).[22] Also the peptide with Phe(*p*-guanidine) appeared to be the most active substrate of cathepsin G.[21] As already mentioned, canonical inhibitors interact with cognate enzyme in a substrate-like fashion. The good correlation between substrate and inhibitory activity of peptides modified in P_1 position by Phe derivatives suggests that introduction into the peptide chain of non-natural amino acids did not influence the mechanism of inhibition of the SFTI-1 analogues studied.

In summary, we would like to point out that synthetic Phe derivatives introduced in the inhibitor P_1 position are well tolerated and in the case of chymotrypsin some of the modifications gave extremely potent inhibitors.

3. Materials and methods

3.1. Peptide synthesis

All the peptides were synthesized by the solid-phase method using Fmoc chemistry. The following amino acid derivatives were used: Fmoc-Gly, Fmoc-Abu, Fmoc-Arg(Pbf), Fmoc-Cys(Trt), Fmoc-Thr(*t*Bu), Fmoc-Ser(*t*Bu), Fmoc-Ile, Fmoc-Pro, Fmoc-Phe, Fmoc-Asp(O*t*Bu), Fmoc-Phe(*p*-NO_2), Fmoc-Phe(*p*-F), Fmoc-Phe(*p*-NH_2), Fmoc-Phe(*p*-CH_3), Fmoc-Phe(*p*-guanidine) and Fmoc-Pal. The C-terminal amino acid residue, Fmoc-Asp(O*t*Bu), was attached to the 2-chlorotritylchloride resin (substitution of Cl 1.46 mequiv/g) (Calbiochem-Novabiochem AG, Switzerland) in the presence of an equimolar amount of DIPEA based on the amino acid in anhydrous condition in DCM solution. Peptide chains were elongated in the consecutive cycles of deprotection and coupling. Deprotection was performed with 20% piperidine in DMF/NMP (1:1, v/v) with addition of 1% Triton X-100, whereas the chain elongation was achieved with standard DIC/HOBt chemistry; 3 equiv of protected amino acid derivatives were used. Nphe(*p*-NO_2) was introduced into the peptide chain by the submonomer approach.[23] In the first step, bromoacetic acid (5 equiv) was attached to the peptidyl-resin using DIC/HOBt method, followed by nucleophilic replacement of bromine with *p*-nitrobenzylamine (8 equiv). After completing the syntheses, the peptides were cleaved from the resin simultaneously with the side chain deprotection in a one-step procedure, using a TFA/phenol/triisopropylsilane (88:5:2.5, v/v/v) mixture.[24] In the last step, the disulfide bridge formation was performed using a 0.1 M methanolic iodine solution and the procedure described elsewhere.[25] The crude peptides were purified by HPLC on a Beckman Gold System (Beckman, USA) using an RP Kromasil-100, C_8, 5 μm column (8 × 250 mm) (Knauer, Germany). The solvent systems were 0.1% TFA (A) and 80% acetonitrile in A (B). Either isocratic condition or linear gradient were applied (flow rate 3.0 mL/min, monitored at 226 nm). The purity of the synthesized peptides was checked on an RP Kromasil 100, C_8, 5 μm column (4.6 × 250 mm) (Knauer, Germany). The solvent systems were 0.1% TFA (A) and 80% acetonitrile in A (B). Linear gradient from 10% to 90% B for 30 min, flow rate 1 mL/min, monitored at 226 nm. The mass spectrometry analysis was carried out on a MALDI MS (a Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany) using α-CCA matrix.

3.2. Determination of the association equilibrium constants

The bovine β-trypsin (Sigma Chem. Co. USA) concentration was determined by spectrophotometric titration with 4-nitrophenyl-4′-guanidinobenzoate (NPGB) at an enzyme concentration oscillating around 10^{-6} M.[26] A standardized trypsin solution was used to titrate ovomucoid from turkey egg whites, which in turn served to determine the solution concentration of the bovine α-chymotrypsin (Sigma Chem. Co. USA). A bovine β-trypsin standardized solution of sunflower trypsin inhibitor (SFTI-1) was used for the determination of the cathepsin G (Biocentrum Sp. z o.o., Kraków, Poland) active form. The concentrations of the SFTI-1 analogues were determined by titration of their stock solutions with standardized bovine α-chymotrypsin and cathepsin G, with Suc-Ala-Ala-Pro-Leu-4-nitroanilide and Ac-Phe-Ala-Thr-Phe(4-guanidine)-ANB-NH_2 as substrates,[21] respectively. The association constants were measured by the method developed in the laboratory of Laskowski, Jr.[27,28] Enzyme–inhibitor interactions were determined in the 0.1 M Tris–HCl (pH 8.3) buffer containing 20 mM $CaCl_2$ and 0.005% Triton X-100 at room temperature. Increasing amounts of the inhibitor, varying from 0 to $2E_0$ (E_0—the initial enzyme concentration), were added to a fixed amount of the enzyme. After a 3-h incubation, the residual enzyme activity was measured on a Cary 3E spectrophotometer (Varian, Australia) using the turnover substrate. The measurements were carried out at initial enzyme concentrations over the ranges 5.3 and 8.0 nM for chymotrypsin and cathepsin G, respectively. The residual enzyme activity was measured with Z-Phe-Ala-Thr-Tyr-ANB-NH_2[29] and Ac-Phe-Ala-Thr-Phe(4-guanidine)-ANB-NH_2 as chromogenic substrates for chymotrypsin and cathepsin G inhibitors, respectively. In all cases the initial substrate concentration was below 0.1 K_M. The experimental points were analysed by plotting the residual enzyme concentration [*E*] versus the initial inhibitor concentration [I_0]. The experimental data were fitted to the theoretical values using the GRAFIT software package.[30]

3.3. Proteolytic susceptibility

The SFTI-1 analogues were incubated in 100 mM Tris–HCl buffer (pH 8.3) containing 20 mM $CaCl_2$ and 0.005% Triton X-100 using catalytic amounts of the enzymes (1 mol %).[31] The incubation was carried out at room temperature and sample aliquots of the mixture were taken out periodically and submitted to RP-HPLC analysis. The analysis were performed on an HPLC Gold System (Beckman, USA) using a Vydac Protein & Peptide, C_{18}, 10 μm column (4.6 × 250 mm) (Grace, USA). The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). Linear gradient from 10% to 90% B for 40 min, flow rate 1 mL/min, monitored at 226 nm.

3.4. Serum stability assay

Human serum (500 μL) collected from healthy volunteer donors (kindly provided by Dr. Julia Kulczycka, Laboratory of Clinical Immunology Medical University of Gdansk) was mixed with 500 μL of 0.2 M Tris–HCl buffer (pH 8.3), placed into Eppendorf tube, followed by addition of 10 μL of peptide (2 mg/mL). After 1 h, 4 h and 24 h the 50-μL aliquots of the mixture were analyzed by HPLC. To confirm the proteolytic activity, the serum was incu-

bated with chromogenic substrates recently developed by our group. Ac-Phe-Val-Thr-Phe(4-guanidine)-ANB-NH_2,[21] Abz-Tyr-Tyr-Abu-ANB-NH_2[32] and Z-Phe-Phe-Pro-Val-ANB-NH_2[29] were used to determine cathepsin G, proteinase 3 and human leukocyte elastase, respectively. The analysis was performed on an HPLC Gold System (Beckman, USA) using a Vydac Protein & Peptide, C_{18}, 10 μm column (4.6 × 250 mm) (Grace, USA). The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). Linear gradient from 10% to 90% B for 40 min, flow rate 1 ml/min, monitored at 226 nm.

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