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Design and X-ray crystal structures of human thrombin with synthetic cyanopeptide-analogues

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Based on the X-ray crystals of cocrystallized cyanopeptide-trypsin and cyanopeptide-thrombin-complexes, a rational drug design succeeded in the establishment of suitable lead structures for the development of new potential inhibitors of thrombin. This report deals with the design and X-ray crystallography data of new synthetic, low-molecular weight cyanopeptide-analogues, RA-1008 and RA-1014, complexed with human α-thrombin at 1.85 Å resolution. The crystal structures of the complexes reveal, by analogy with modeling studies, that the salt bridge of Asp189 to this type of synthetic thrombin inhibitors leads to an almost identically binding into the S1 specificity pocket in comparison to the complex of the natural products, whereas in the overall binding modes the P2–P4 substructures differ from those of the leads. The strongest member of the second series of described thrombin inhibitors, RA-1014, shows in the crystal complex with thrombin a slightly higher affinity towards the enzyme than RA-1008 as confirmed by inhibition tests. This result and other key informations will be helpful to design a more potent series of inhibitors.

1. Introduction

Secondary metabolites of blue-green algae (cyanobacteria) have been widely screened in search for new lead compounds in structure-activity relationship studies. Cyanopeptides are major metabolites of cyanobacteria, and as their name implies, are of peptidic character. Besides hepatotoxic and neurotoxic properties, cyanopeptides show a broad spectrum of biological activities (Radau 2000; Namikoshi and Rinehart 1996; Yasumoto and Satake 1998; Moore 1996; Sings and Rinehart 1996), including antitumor (Trimurtulu et al. 1994), immunosuppressive (Zhang et al. 1997), and antimicrobal effects (Pergament and Carmeli 1994) as well as angiotensin-converting enzyme inhibitory action (Okino et al. 1993) and cardioactive effects (Moore et al. 1989).

Many of the isolated natural compounds of non-toxic cyanobacteria strains inhibit trypsin-like serine proteases, which play pivotal roles in the human organism. Failures of one or more of these enzymes may cause a state of imbalance between protease and antiprotease (endogenous protease inhibitors, serpins) and may lead to an excess of proteolytic activity and/or to the development of diseases such as stroke, heart failure, deep vein thrombosis, and other thromboembolic effects.

The discovery and development of oral inhibitors of the above mentioned enzymes therefore presents a notable approach for improving the treatment of these disorders and remains a challenge for medicinal chemists.

Active site thrombin inhibition still remains a very actual area of antithrombotic research because of the need of oral available inhibitors and the withdraw of ximelagatran, the first oral thrombin inhibitor, from the market.

2. Investigations, results and discussion

2.1. X-ray structural properties of the cyanobacterial lead compounds

A rational drug design process based on the X-ray crystal of the cocrystallized trypsin–aeruginosin 98-B (**1**)–complex (Sandler et al. 1998) and the cocrystallized thrombin–aeruginosin 298-A (**2**)–complex (Steiner et al. 1998), respectively, resulted in new potential inhibitors of thrombin. Aeruginosin 98-B (**1**, Fig. 1) and aeruginosin 298-A (**2**) were isolated from *Microcystis aeruginosa* [NIES-98 (Murakami et al. 1995) and NIES-298 (Murakami et al. 1994), respectively; Microbial Culture Collection of the National Institute for Environmental Studies, Japan]. All members of the aeruginosin family contain the unusual α-amino acid unit 2-carboxy-6-hydroxy-octahydroindole (Choi), the 4-hydroxyphenyllactic acid (Hpla) side-chain, and the basic arginine-derived side-chain as common amino acid or peptidomimetic components, respectively (Radau 2005). Structural variations mainly occur in position P3 [P/S-nomenclature according to Schechter (Schechter and Berger 1967)].

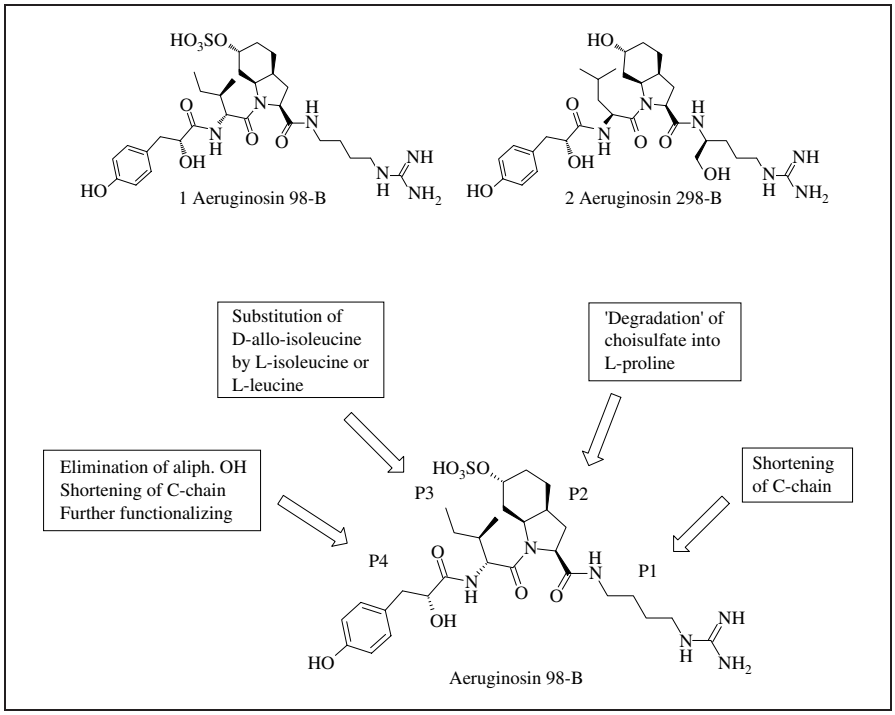

Fig. 1:
Structures of cyanobacterial lead structures and modification scheme

X-Ray measurements of the cocrystallized trypsin-**1**-complex (Sandler et al. 1998) reveal the lack of any interactions of **1** with trypsin's catalytic triad (Ser195, His57, Asp102). The Ser195 residue does not show covalent bonds or other close contacts to any atoms of **1**. Therefore, aeruginosin 98-B's ability of inhibiting trypsin-like serine proteases obviously can be interpretated as a sum of several hydrogen bonds and van der Waals interactions between inhibitor and enzyme. These interactions still allow them to act as strong and reversible inhibitors, whereas other natural products like cyclotheonamide A, another cyclic peptide isolated from the marine sponge of the genus *Theonella,* bind in a covalent manner to Ser195 (formation of a hemiacetal) of the catalytic triad of trypsin-like serine proteases (Lee et al. 1993; Lewis et al. 1993). In each aeruginosin, the basic side-chain in position P1 is essential for inhibiting the trypsin-like serine proteases. The terminal guanidino group fits perfectly into the S1 pocket of trypsin or thrombin building a salt bridge with Asp189. Each of the three other building blocks in positions P2−P4 of the inhibitors contributes to the interactions of the aeruginosin derivatives with these enzymes. Even the distant Hpla moiety in position P4 of **1** shows a strong water-mediated interaction between the phenolic hydroxyl group and the amino acids Cys220 and Ser146 of trypsin (Sandler et al. 1998). In addition, the substitution pattern of the phenolic partial structure – namely the kind of halogen atom in ortho-position to the hydroxyl group – influences the strength of inhibition. Bromination and dihalogenation of **1** seem to level the inhibitory property of these cyanopeptides (Ishida et al. 1999).

2.2. Design of synthetic analogues

With the objective not to allow covalent interactions between ligand and catalytic triad, agmatine was retained as the basic component in P1 (Fig. 1). In position P2 the Choi heterocycle was replaced with L-proline for three reasons: Firstly, L-proline is absolutely needed as a conformational discriminator to imitate the characteristically curved conformation of the aeruginosins by analogy with Choi. Secondly, the synthesis of the Choi subunit would be more time-consuming than the use of L-proline (for the synthesis of Choi derivatives see Valls et al. 2001 and 2002; Bonjoch et al. 1996; Fukuta et al. 2004). Finally, this modification enables the connection of L-proline with L-isoleucine (Radau et al. 2003; Radau and Stürzebecher 2002) without any fear of steric conflicts between L-isoleucine and Choi described in literature (Sandler et al. 1998). Furthermore, the replacement of Hpla with 3-(4-hydroxyphenyl)propionyl and benzoyl moieties as well as phenylmethanesulfonyl and phenylacetyl partial structures has recently been reported (Radau et al. 2003; Radau and Stürzebecher 2002). These proposed structural modifications led to the syntheses of aeruginosin analogues RA-1001, RA-1002, RA-1003, RA-1004, RA-1008, RA-1014, and finally RA-1013 (Fig. 2). Practical synthetic routes to these novel inhibitors were designed with synthetic ease in mind and with the added goal to minimize or eliminate racemisation (Radau et al. 2003).

2.3. Inhibition properties of the new inhibitors

Compounds RA-1001 and RA-1002 are potent inhibitors of thrombin with K_i values of 5.6 μM and 8.7 μM, respectively (Table 1). In contrast to RA-1001, aeruginosin 98-B (1) is a more potent inhibitor of trypsin than thrombin (IC_{50} 0.6 and 10.0 μg/mL respectively). The reason for this different behaviour may be explained by steric conflicts between aeruginosin's bulky Choi group in position P2 and the S2 pocket of thrombin (60's loop), in particular in **1** there is a sulphated hydroxyl function, which is less prominent in the L-proline moiety of RA-1001. Surprisingly, the synthetic precursor of RA-1001, the primary amine RA-1003, inhibits thrombin in the same order of magnitude (K_i 9.0 μM). None of the four analogues of the first series (RA-1001, RA-1002, RA-1003, RA-1004) decreases the activity of plasminogen activator urokinase (uPA), factor Xa, and human mast cell tryptase.
Compound RA-1008 shows a satisfactory inhibition of thrombin, but is also a reasonable trypsin inhibitor. Comparing the structural features of RA-1008 with those of RA-1001, the phenylmethanesulfonyl moiety in P4 may

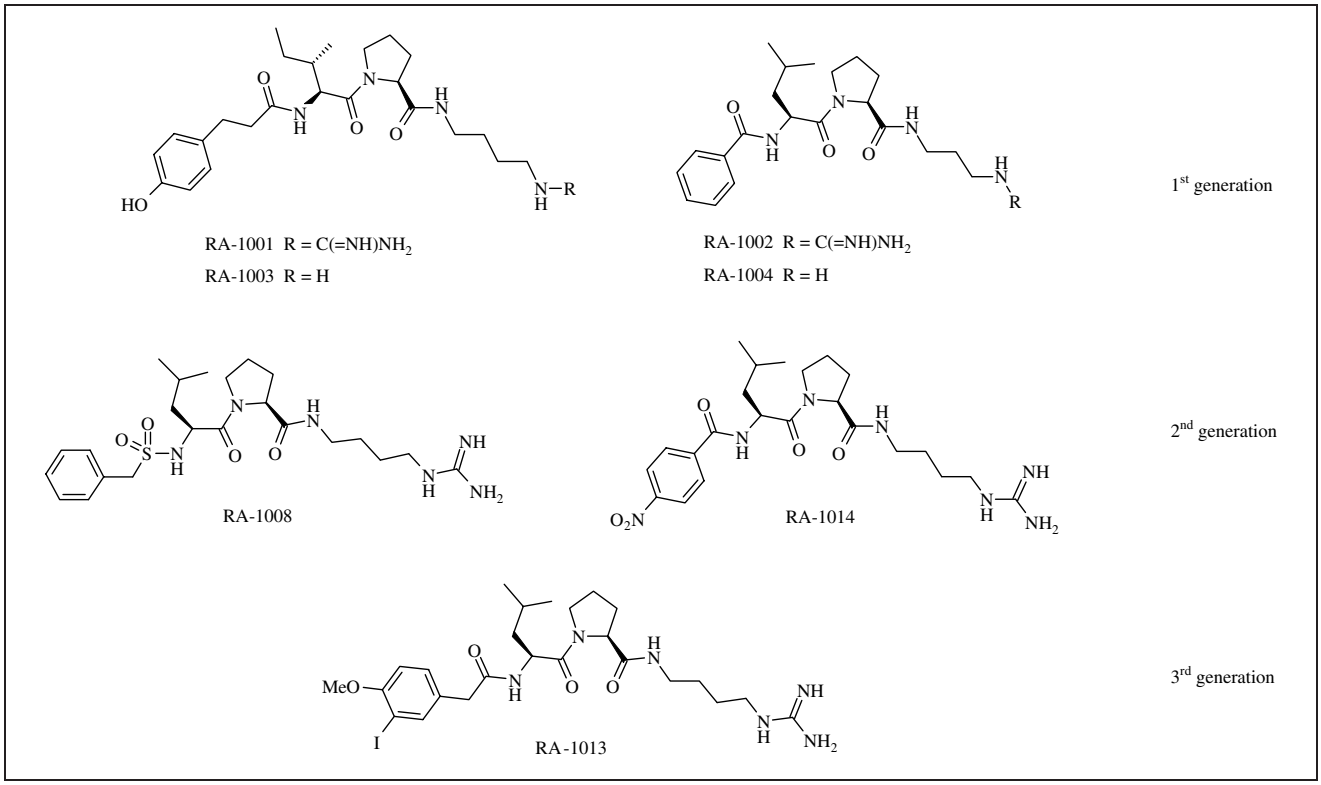

Fig. 2: Three generations of synthetic aeruginosin analogues

Table 1: IC_{50}-values of aeruginosin 98-B (1) and aeruginosin 298-A (2); Ki-values of synthetic aeruginosin-analogues

Nr.	K_i (μM) (n = 3)					
	Trypsin	Thrombin	u-PA	Factor Xa	Tryptase	Plasmin
RA-1001	32	5.6	>1000	>1000	>1000	–
RA-1002	>1000	8.7	>1000	>1000	>1000	–
RA-1003	>1000	9.0	>1000	>1000	>1000	–
RA-1004	>1000	62	>1000	>1000	>1000	–
RA-1008	5.4	10.6	>1000	>1000	–	–
RA-1013	>1000	15	>1000	72	–	>1000
RA-1014	10.9	1.9	>1000	250	–	>1000
	IC_{50} (μg/mL)					
1	0.6	10.0	–	–	–	7.0
2	1.0	0.3	–	–	–	–––

– not determined, ––– no inhibition

contribute to the stronger inhibition of trypsin. For the inhibitory consequences of an exchange of L-isoleucine by L-leucine see Radau et al. (2003b).

On the one hand compound RA-1014 represents the most active member of the new series of thrombin inhibitors, while on the other hand there is the shortcoming of less selectivity (K_i 10.9 μM [trypsin]; 250 μM [factor Xa]).

The most promising results were obtained from the halogenated derivative RA-1013. The P4 moiety of this inhibitor contains a iodinated phenylacetic acid that represents a structural compromise between the phenylpropionic acid substructure of RA-1001 and RA-1003 and on the other hand the benzoylated substructures of RA-1002 and RA-1004. The very interesting and surprising aspect of this inhibitor is the remarkable assignment of the partial structures of RA-1013 to the subsites S1–S4 of thrombin *in silico* (unpublished data). The (3-iodo-4-methoxyphenyl)acetyl unit (P4) and not the P1/P2 region interacts with the catalytic center of thrombin because the iodine atom fits exactly into a triangle opened up from the residues His57 and Ser195 of the catalytic triade and the Cys42-Cys58 disulfide bridge. This interaction disturbs the catalytic activity of the enzyme very drastically. Unfortunately the efforts to confirm this first hint towards a presumably new mode of inhibition failed in an attempt to crystalize the RA-1013–thrombin complex.

2.4. X-ray crystal studies of RA-1008 and RA-1014

Diffraction data of the complexes of human α-thrombin (283 residues) with the inhibitors RA-1008 and RA-1014 were collected with an in-house source, and the observed crystal structure was refined to a resolution of 1.85 Å with a final R_{cryst} and R_{free} of 19.5% and 26.3%, respectively for RA-1008 and a final R_{cryst} and R_{free} of 19.1% and 26.0%, respectively for RA-1014 (for data collection and refinement statistics see Table 2). The mean temperature factors for the whole protein of 26.4 $Å^2$ for RA-1008 and

Table 2: Data collection and refinement statistics

	Thrombin RA-1008	Thrombin RA-1014
PDB code	1YPL	1YPM
Data collection		
Temperature (K)	103	103
No. of crystals	1	1
Resolution range (Å)	50.0–1.85	50.0–1.85
Space group	C2	C2
Unit cell (Å, ° for ß)	a = 71.1 b = 71.6 c = 72.2 β = 100.4	a = 69.7 b = 71.7 c = 71.3 β = 99.7
No. of observations	87351	64512
No. of unique reflections	28802	27275
Completeness (%)	99.4 [99.9]	94.9 [91.6]
Mean I/σ	9.7 [2.7]	15.7 [3.2]
R_{sym} (%)	9.9 [44.4]	5.3 [31.5]
Refinement statistics		
Refined residues	277	277
Refined Ligand atoms	34	34
Refined water molecules	191	155
Resolution range in refinement (Å)	10.0–1.85	10.0–1.85
R_{cryst} ($F_o > 4\sigma F_o$; F_o)	19.5	19.1
R_{free} ($F_o > 4\sigma F_o$; F_o)	26.3	26.0
Rmsd bond lengths (Å)	0.017	0.017
Rmsd bond angles (°)	2.2	1.9
Average B value (Å^2)	26.4	30.6
Main chain (Å^2)	23.6	27.5
Side chain (Å^2)	29.2	33.6
Ligand (Å^2)	27.5	46.1
Waters (Å^2)	32.3	33.4
Ramachendran plot ||		
Most favoured (%)	85.6	87.2
Additional allowed (%)	14.4	12.3
Generously allowed (%)	0.0	0.4
Disallowed (%)	0.0	0.0

30.6 Å^2 for RA-1014 are in the same range as the already published complex of aeruginosin 298-A (PDB entry: 1A2C). The B-factors of the inhibitors differ significantly (RA-1008: 27.5 Å^2, RA-1014: 46.1 Å^2). In comparison with the B-factor of aeruginosin 298-A (32.4 Å^2) the value for RA-1008 is slightly lower. The aromatic residue is primarily responsible for the high average B-factor observed for RA-1014. This indicates a higher flexibility of this part of the molecule. The final model shows the typical two domains of the antiparallel β-type as described in literature (Branden and Tooze 1991). The $F_{obs}-F_{calc}$ electron density (σ-level 2.5) maps clearly show the location of both inhibitors (Fig. 3). The only exception is the nitro group of the inhibitor RA-1014 which is poorly defined.

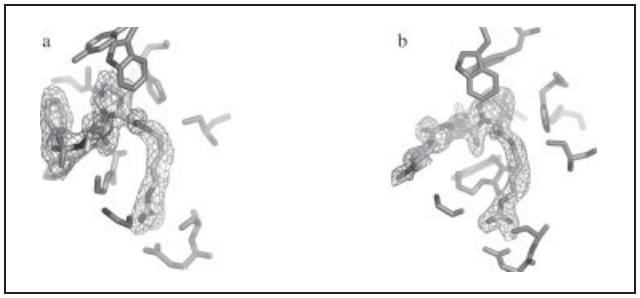

Fig. 3: Experimental Fobs-Fcalc electron densities of RA-1008 (a) and RA-1014 (b)

RA-1008 and RA-1014 occupy the active site of thrombin, with the guanidino group present in the S1 specificity pocket containing the residues Asp189, Ala190, Gly216, Gly219 as well as Trp215 (Fig. 4). An essential prerequisite for this interaction is the binding of the protonated guanidino moiety (NH1, NH2 of the agmatine residue) to the carboxylate of Asp189 forming a nearly symmetric salt bridge with distances of 2.5 Å/2.8 Å for RA-1008 and 2.6 Å/2.7 Å for RA-1014. The binding mode is further stabilised by a hydrogen bond to the carbonyl group of Gly219. This hydrogen bond at a distance of 3.0 Å for RA-1008 is a little stronger than in the complex with RA-1014 (3.2 Å). In the case of RA-1008 the ε-nitrogen atom of the agmatine residue forms an additional hydrogen bond to a water molecule. Furthermore, in both structures the backbone nitrogen of the agmatine moiety of RA-1008 and RA-1014 forms a hydrogen bond to the hydroxyl group of Ser214. In both inhibitors this hydrogen bond has the same distance of 2.9 Å. A short β-ladder is formed by hydrogen bonds between the leucine nitrogen and carbonyl group of the proline ring of RA-1008 on one side and the backbone oxygen and nitrogen of Gly216 on the other side. The hydrogen bond to the backbone oxygen of Gly216 is missing in the complex of thrombin with RA-1014. In this case the contact distances between RA-1008 and thrombin are 3.1 Å and 3.3 Å for the two hydrogen bonds. For the complex with RA-1014 there is only one weak hydrogen bond with a distance of 3.6 Å. The sulfonyl spacer fixes the terminal benzyl group of RA-1008 in a completely different orientation compared to RA-1014 between the S2 and the S3 pocket. This leads to an orientation of the sulfonyl group pointing away from the protein surface into the solvent.

Interestingly, the solvent structures of both complexes differ from each other. In the structure of RA-1008, three water molecules that formed hydrogen bonds to the inhibitor were found whereas no electron density for a water molecule forming a hydrogen bond to the inhibitor was found in the structure of RA-1014.

Compared with aeruginosin 298-A, the inhibitors RA-1008 and RA-1014 form less hydrogen bonds. Aeruginosin 298-A interacts through two additional hydrogen bonds with both hydroxyl functions (Argol and Hpla) to His57 and Gly219, respectively. Most of the other common hydrogen bonds in all three complexes are found with aeruginosin 298-A to be between 0.1 and 0.6 Å shorter than in those of the synthetic analogues.

In total, there are 71 van der Waals contacts between RA-1008 and thrombin and 81 van der Waals contacts between RA-1014 and the enzyme. The superimposition, which has been performed with the program ProFit 2.3 (McLachlan 1982), of both structures with the already published complex of aeruginosin 298-A shows a rmsd of 0.38 Å for RA-1008 and 0.39 Å for RA-1014 for the overall structure. However, significant differences between the binding modes of these inhibitors can be seen (Fig. 5). Interestingly, they do not differ in the binding to the S1 specificity pocket. Extended differences are observed for the amino acids of the 60's loop (rmsd between 0.18 Å and 1.12 Å). In addition, the residues of the catalytical triad (His57, Asp102, Ser195) seem to be quite flexible.

2.5. Conclusions

Cyanopeptides represent a broad spectrum of inhibitors of trypsin-like serine proteases. Depending on the amino acid sequences, conformations, and configurations, these compounds are able to inhibit various (trypsin-like) serine pro-

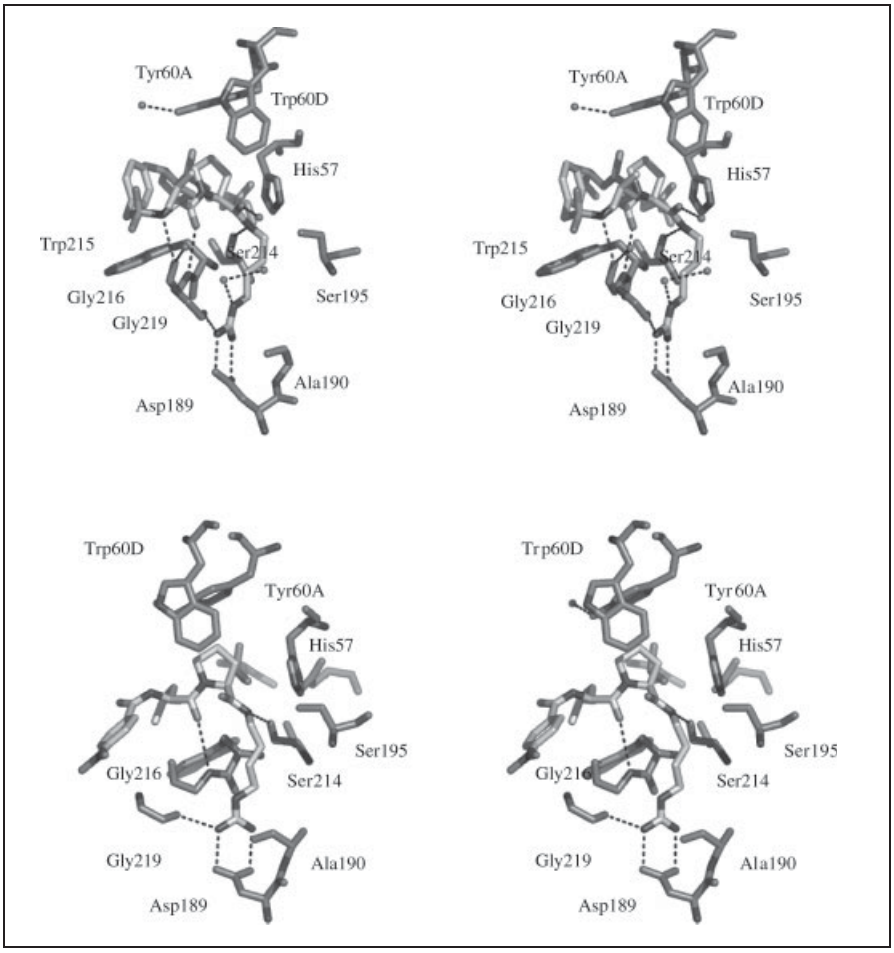

Fig. 4:
Stereoviews of RA-1008 (a) and RA-1014 (b) complexed with the catalytic site of human thrombin. The hydrogen bonds are represented as dashed lines

teases selectively. Many more cyanopeptides with interesting biological activities will likely be isolated in continued screening programs in the near future. Because of the richness and diversity of these bioactive substances, it should be possible to find more new lead structures with novel mechanisms of action for the rational drug design of potent, selective acting inhibitors.

Herein, we described the design and X-ray crystal structures of the new cyanopeptide-derived thrombin inhibitors RA-1008 and RA-1014 complexed with human α-thrombin at 1.85 Å resolution. The crystal structures of RA-1008 and RA-1014 reveal that the salt bridge of Asp189 to this type of synthetic thrombin inhibitors leads to an almost identically binding into the S1 specificity pocket in comparison to the complex of the lead structure and natural thrombin inhibitor aeruginosin 298-A. However, the overall binding modes of the synthetic inhibitors (P2−P4 substructures) show not negligible differences. Accordingly, we assume the binding mode of RA-1014 is somewhat better because of the slightly higher affinity towards thrombin. The sulfonyl group seems to force the benzyl group of RA-1008 into a less favourable orientation, preventing the assembling of close contacts to the protein.

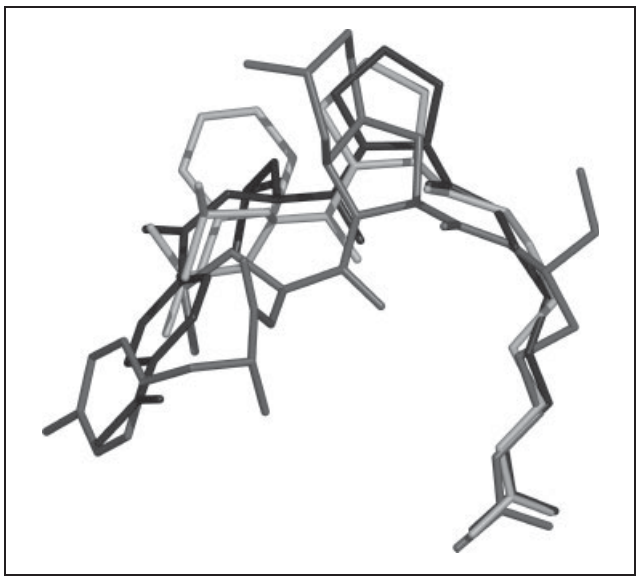

Fig. 5: Alignment of inhibitors RA-1008, RA-1014, and aeruginosin 298-A

The result of this study represent the basis for the design of a more potent third generation of inhibitors. Further investigations on structure-activity relationships are in progress in order to complete the results of this study. Firstly, the efforts are focused on the transfer of the guanidino-derivatives into hydroxyguanidino-derivatives (prodrug concept) in order to enhance oral bioavailability, and secondly on the syntheses of derivatives of RA-1013 in order to get more informations about the proposed new binding mode of the P4 residue with the catalytic triad.

3. Experimental

3.1. Crystallisation

Human α-thrombin was purchased from Haemochrom, Essen, D, and acetylhirudin (54–56) (sulfated) from Bachem, Bubendorf, CH.

Inhibitors were cocrystallized with hirudin-human-α-thrombin at 4 °C by the hanging-drop vapour diffusion method. The experiments were peformed by using 22–27% polyethylene glycol 8000 and 0.1 M sodium phosphate buffer (pH 7.5) as reservoir solution. Human thrombin was concentrated to 4–5 mg/mL, incubated with hirudin solution (50 mg/mL), and the inhibitor

concentrations varied between 2 and 3 mM. Crystals of thrombin grew within a few days as micro crystals. By application of the macroseeding technique lager crystals were obtained that diffracted 2 Å or better.

3.2. Data collection

For data collection, appropriate crystals were cryopreserved by using the reservoir solution as cryoprotectant and flash-frozen in liquid N2. The data set was collected at cryo conditions (100 K) with 5 min exposure in 0.5° oscillation steps per frame, 120 mm detector to crystal distance and with CuK_α radiation ($\lambda = 1.5418$ Å) using a Rigaku RU-300 rotating anode generator at 50 kV and 90 mA equipped with focusing mirrors (MSC, USA) and an R-AXIS IV++ image plate system. The crystal belonged to the monoclinic space group C2 with unit cell dimensions shown in Table 2. One monomer in the asymmetric unit with a Matthews coefficient of 3.0 Å^3/Da and 59.0% solvent content for RA-1008 and 2.9 Å^3/Da and 57.8% for RA-1014. All data were processed and scaled with the programs DENZO and SCALEPACK implemented in the HKL2000 package (Otwinowski and Minor 1997). Data collection statistics are summarised in Table 2. The hirugen-human-α-thrombin complex structure determined previously (Obst et al. 2000) was used as starting model for thrombin in order to determine the structure by molecular replacement with the program CNS (Brünger et al. 1998).

3.3. Model building and crystallographic refinement

Conventional crystallographic refinement (rigid body, positional and temperature factor) was done with the CNS program package. The model building was carried out using the program O (Jones et al. 1991). The structure was gradually improved by crystallographic refinement with the program SHELXL (Sheldrick and Schneider 1997). Here, at least 10 cycles of conjugate gradient minimization were performed with default restraints on bonding geometry and B-values. Five percent of all data were used for R_{free} calculation. Amino acid side-chains were fitted into sigma-A-weighted $2F_o-F_c$ and F_o-F_c electron density maps using O. Restraints were applied to bond lengths and angles, chiral volume, planarity of aromatic rings and van der Waals contacts. During the last refinement cycles, riding H atoms were introduced without using additional parameters. In the final structure, the amino acid residues 148–149E are poorly defined in the electron density, and could not be included into the structure. The quality was assessed by calculation of the Ramachandran plot with the program PROCHECK (Laskowski et al. 1993). Accordingly, 85.6% and 87.2% of the residues are found in the most-favored regions, none is flagged as an outlier. A summary of the final model and refinement statistics is given in Table 2. The figures were prepared with Isis Draw and Pymol (DeLano 2004).

3.4. Protein Data Bank accession numbers

The atomic coordinates of the thrombin structures in complex with the inhibitors have been deposited with the Brookhaven Protein Data Bank under the following entry codes: 1YPL (RA-1008) and 1YPM (RA-1014), respectively.

3.5. Determination of enzyme kinetics

Inhibition tests were carried out to published protocol (Stürzebecher et al. 1997). The K_i values reported are means from at least three determinations.

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