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ScienceDirect
European Journal of Medicinal Chemistry 43 (2008) 1330–1335
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Short communication

Structure of a novel thrombin inhibitor with an uncharged D-amino acid as P1 residue

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Received 1 November 2005; received in revised form 22 July 2007; accepted 26 July 2007
Available online 9 September 2007

We would like to dedicate this work to Prof. Dr. Jörg Stürzebecher, one of the pioneers in modern thrombin inhibitor research.

Abstract

Thrombin, the ultimate proteinase of the coagulation cascade, is an attractive target for the treatment of a variety of cardiovascular diseases. Previously, a series of novel thrombin inhibitors, discovered by employing a powerful and new computer-assisted multiparameter optimization process (CADDIS®), have been synthesized. We have now crystallized the complex of human α-thrombin with the most potent of these inhibitors, 8-5 ($K_i = 3$ nM), and have determined its 2.3 Å X-ray crystal structure. The Fourier map displayed clear electron density for the inhibitor. The central part of the inhibitor binds in an improved melagatran-like mode, while the structure identifies a D-tyrosine as P1 residue which forms a charged hydrogen bond with Asp 189 of thrombin. This is the first crystal structure of a thrombin–inhibitor complex, where an uncharged inhibitor residue makes hydrogen bonds within the thrombin S1 pocket. Additionally, novel favourable intermolecular hydrogen bonds of the inhibitor with the thrombin backbone become possible due to the D-configuration of the P1 residue. Two flanking voluminous side chains increase the strength of the subjacent hydrogen bonding system by shielding it from the bulk solvent.

Keywords: Blood coagulation; Haemostasis; Serine proteinase; Thrombin; Inhibitor

The serine proteinase thrombin plays a pivotal role in thrombosis and haemostasis, catalyzing several procoagulant processes which lead to clot formation [1]. Thrombin has therefore been implicated in various disease processes such as myocardial infarction, stroke and pulmonary embolism. A huge number of potent small synthetic thrombin inhibitors have been designed, synthesized and tested in the search for orally active anticoagulants [2,3]. However, despite enormous efforts, so far there has not been much success in making them available for therapeutic application [3]. In order to find novel types of thrombin inhibitors, a new computer-assisted drug discovery approach [4] has recently been applied to identify a novel series of promising thrombin inhibitors [5]. The identified lead compounds exhibited thrombin inhibitory constants in the lower nanomolar range. They are by far the most selective synthetic thrombin inhibitors with selectivities greater than 10^5-fold towards other proteinases such as factor Xa, factor XIIa, urokinase, plasmin and plasma kallikrein. Furthermore, these compounds exhibit a favourable profile comprising non-toxicity, high metabolic stability, low serum protein binding, good solubility, high anticoagulant activity and a slow and exclusively renal elimination from the circulation in a rat model.

To conceive the structural basis for these striking properties, we have crystallized [6] human α-thrombin in space group *C*2 and in presence of the inhibitors benzamidine (Bz)

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0223-5234/$ - see front matter © 2007 Published by Elsevier Masson SAS.
doi:10.1016/j.ejmech.2007.07.019

and hirugen (sulfated hirudin 56-63), a peptide derived from the leech-derived thrombin inhibitor hirudin, which specifically targets the fibrinogen binding exosite (exosite I) of thrombin (Table 1). For formation of the complex, a 100-fold excess over the determined K_i of inhibitor 8-5 ($K_i = 3$ nM) was soaked into the crystals. After shock-freezing, the crystals diffracted to a maximum resolution of 2.3 Å. With the exception of the autolysis-/148-loop (chymotrypsinogen numbering of thrombin [7]), the main chain of the thrombin molecule was fully defined by electron density, exhibiting a conformation virtually identical with the thrombin–hirugen complex (PDB accession code 1HGT [6]) used for the initial structure solution. The inhibitor could almost fully be traced with the exception of a few atoms in side chains pointing towards the bulk solvent (Fig. 1A), which were only defined by weak electron density. In the following, we will describe the binding mode from the N- to the C-terminus of the inhibitor.

Table 1
Data collection and refinement statistics

Data collection		
Space group		*C*2
Cell constants		
a		70.46
b		70.93
c		72.65
β		100.38
Limiting resolution		2.30 Å
Reflections measured		156 090
R_{merge}: overall, outermost shell (2.30–2.44 Å)		0.057, 0.298
Unique reflections		13 883
Completeness: overall, outermost shell (2.30–2.44 Å)		88.6%, 86.6%
Multiplicity		2.2
Refinement		
Reflections used for refinement		13 734
Resolution range		15.0–2.30 Å
Completeness: overall, outermost shell (2.30–2.42 Å)		87.6%, 78.9%
R value: overall, outermost shell (2.30–2.42 Å)		0.201, 0.232
R_{free}: overall, outermost shell (2.30–2.42 Å)		0.232, 0.287
Test set size		5.1%
RMS standard deviations:	Bond length	0.011 Å
	Bond angles	1.5°
Average *B* value/s.d.		30.7
Ramachandran plot:	Most favoured region	85%
	Favoured region	15%
Non-hydrogen protein atoms		2303
Non-hydrogen inhibitor atoms		150
Solvent molecules		148

Human α-thrombin was bought from Roche Diagnostics (Mannheim, Germany). The synthesis of 8-5 was described elsewhere [5]. A solution (1.5 μl) containing 5 mg/ml human α-thrombin and sixfold molar excess of the sulfated hirudin peptide 55-64 obtained from Bachem (Basel, Switzerland) in 10 mM Tris–HCl pH 8.0, 20 mM NaCl were mixed with 1.5 μl of a reservoir solution consisting of 0.1 M potassium phosphate pH 5.6 and 20% PEG 6000 as precipitant. Monoclinic crystals of space group *C*2 appeared after two-day concentration against the reservoir. The crystals were transferred to the mother solution containing 20% glycerol as cryoprotectant as well as 1 mM inhibitor 8-5, and were mounted after 3 h of soaking under a nitrogen stream (100 K) on a MAR345 image plate system (MAR Research, Hamburg). A complete X-ray data set was taken from a single crystal. The data were processed with MOSFLM, and scaled and loaded using SCALA from the CCP4 program suite (http://www.ccp4.ac.uk/). After rigid body refinement with CNS using the coordinates of the thrombin–hirugen complex (1HGT) [6], the *R*-factor dropped to 31%. The crystallographic refinement was done in several cycles consisting of model building steps performed with MAIN (http://www-bmb.ijs.si/) and conjugate gradient minimization and simulated annealing using CNS (http://cns.csb.yale.edu/v1.1/). Finally, solvent molecules were built in and individual, restrained atomic *B* values were refined. The occupancy of atoms not defined by electron density was set to zero. All main chain angles are located in the most favoured and favoured $\phi\psi$ regions of the Ramachandran plot. The atomic coordinates have been deposited with the protein data base (PDB; http://www.rcsb.org/pdb/) under the code 1XM1.

The N-terminal guanidinylated tranexamic acid (G-Trx, Pos. 1) leans with its cyclohexyl moiety against the rim of the S1 pocket of thrombin (Fig. 2A), made by the backbone of Glu 217 and Gly 219 (Fig. 2B). The terminal guanidinium group is well defined by electron density (Fig. 1A); however, it does not make direct contacts with the carboxylate group of Glu 146, which, in principle, could be reached by the positively charged guanidinium group, nor to water or neighboring protein molecules (Figs. 1B and 2B). Nevertheless, during the initial phase of the binding process the electrostatic potential of Glu 146 might help to guide the inhibitor to the active site region. D-Cyclohexylalanine (D-Cha, Pos. 2), refined in the chair conformation like in a related structure [8], and L-azetidine (L-Aze, Pos. 3), like the D-Chg–L-Aze motif in melagatran [9], slot in the thrombin-characteristic aryl binding site (S4) and the S2 pocket, respectively (Fig. 3). Supported by the sharp turn of the inhibitor backbone enforced by the strained 4-ring L-Aze, as well as by the longer side chain of D-Cha, the latter can reach deeper into the S4 pocket than cyclohexylglycine in melagatran (~1.5 Å) or D-Phe in PPACK[7] (~1 Å), and therefore can better exploit the hydrophobic environment generated by Leu 99, Ile 174 and Trp 215 in this thrombin pocket (Fig. 3). Thus, the D-Cha–L-Aze fragment seems to represent an ideal inhibitory motif for thrombin. The 19-fold increase in K_i upon replacement of D-Cha by D-Phe can be explained by the more rigid architecture of the L-Aze, which forces a strictly planar D-Phe into a position where it would collide with the rim of the pocket, in contrast to the more flexible D-Cha.

It was long assumed that Asp 189 positioned at the bottom of the S1 pocket of thrombin requires a positive countercharge for productive binding. However, Nienaber et al. [10] have crystallized the thrombin complex with 6-fluorotryptamine, an extremely weak thrombin inhibitor ($K_D = 6.9$ mM), which – although a basic compound – is superficially bound in the S1 pocket, but does not form any hydrogen bonds with Asp 189. Malikayil et al. [11] presented a covalent inhibitor, which – although covalent – exhibits a weak potency of only 247 nM and inserts its hydrophobic indole moiety into the S1 pocket, again without contacts to Asp189. In recent years a series of weakly inhibitory D-Cha–L-Pro derivatives [12] lacking a basic moiety have been identified that could very well bind to the S1 pocket by virtue of a neutral moiety; however, no structure has been published so far. In contrast, a series of D-Chg–L-Pro derivatives lead to the identification of highly effective thrombin inhibitors. The structure determination of a 2,5-dichlorobenzyl derivative [13] in complex with thrombin revealed that the uncharged 2,5-dichlorobenzyl moiety establishes hydrophobic contacts with Tyr 228 at the internal wall of the pocket, but

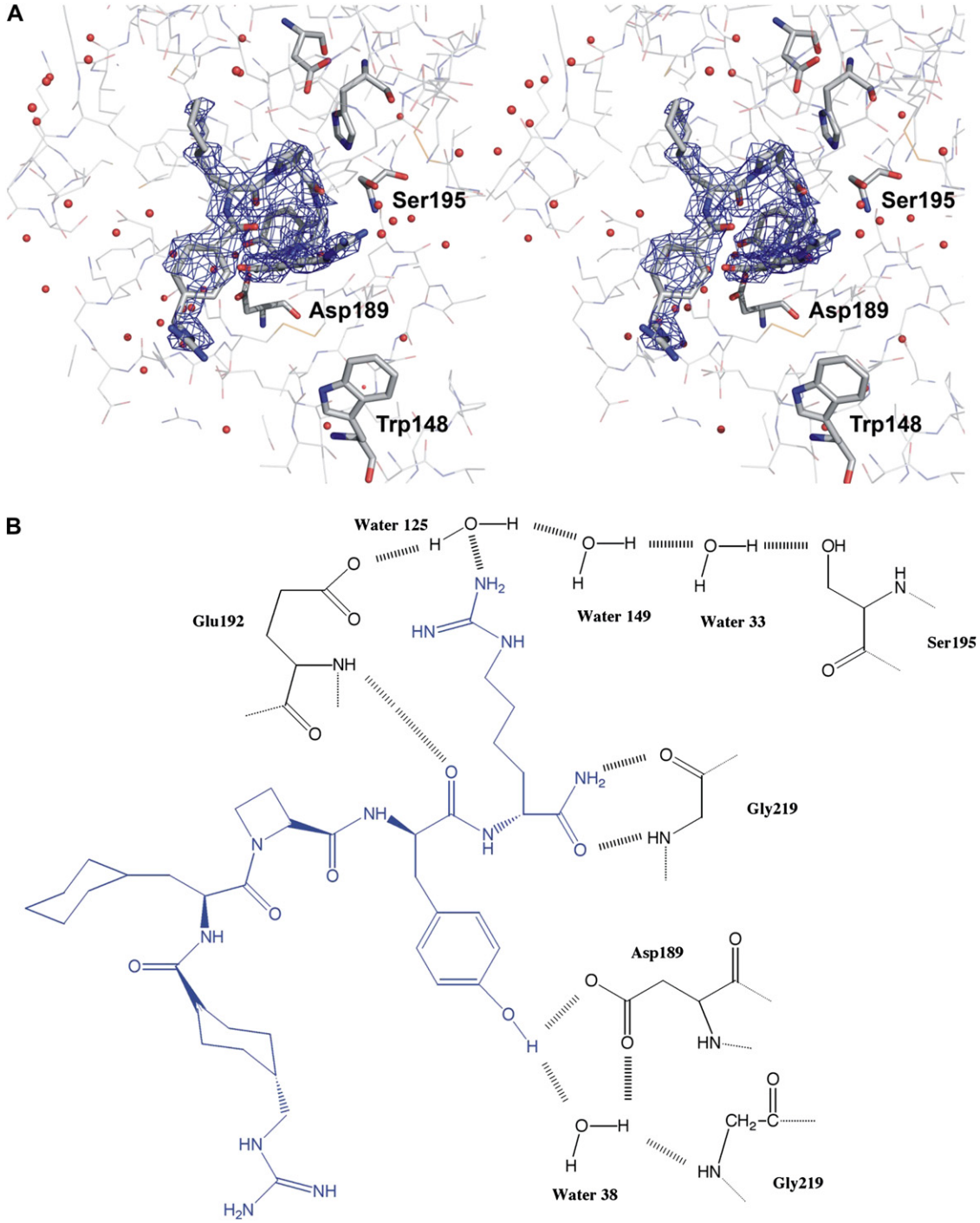

Fig. 1. The unique active site binding mode of the inhibitor 8-5. A. In the composite omit map contoured at 0.75 Sigma, the unusual P1 residue Tyr is very well defined, as most parts of the inhibitor backbone. Also, the electron density of the long homoarginine side chain is sufficiently defined and indicates a tight binding to the active site of thrombin. Important thrombin residues are labeled. B. Schematic drawing of hydrogen bonds between the inhibitor and thrombin active site residues. Important thrombin residues and water molecules are labeled.

does not reach far enough into the S1 pocket to hydrogen bond Asp 189 directly (Fig. 4A). Inhibitor 8-5 instead makes a direct charged hydrogen bond with its D-Tyr OH to one of the carboxyl oxygens of Asp 189 (~2.6 Å; Fig. 1B). Due to the kink at Cβ of the tyrosine side chain, D-Tyr cannot reach into the pocket in the same way as P1-L-Arg, L-Lys, or amidinophenylalanine residues would do. Instead, the phenyl ring of D-Tyr gets closer to the backside of the pocket (Ala 190, Val 213, Trp 215) than to the front edge (Glu 217, Gly 219). Superimposition of the complex structures of melagatran and 8-5 reveals that the aromatic planes of the benzamidine side chain (melagatran) and D-Tyr (8-5) are inclined at an angle of about 60° (Fig. 4B). Nevertheless, the D-Tyr OH occupies a position nearly identical to where one of the guanidyl nitrogens of L-Arg (PPACK; Fig. 4C) or the

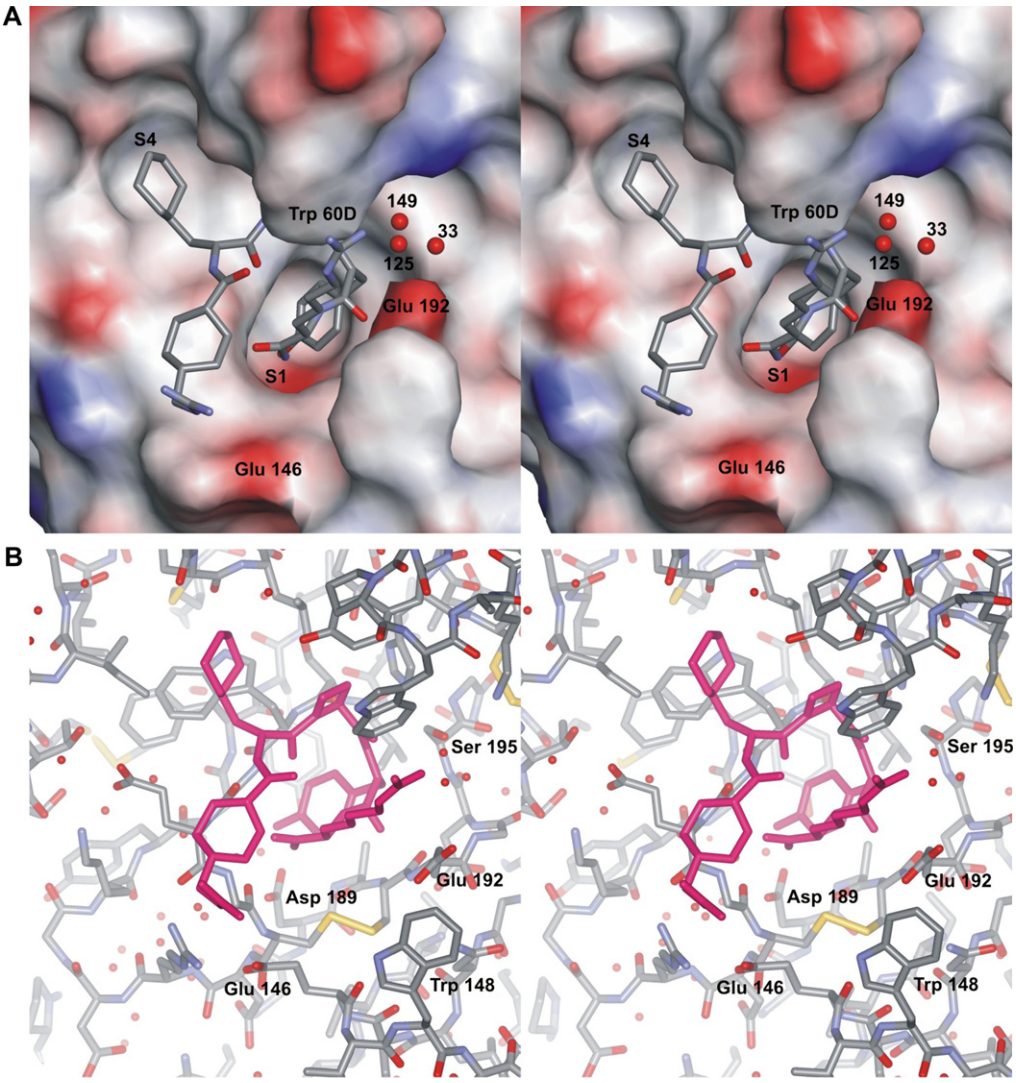

Fig. 2. Active site binding mode of 8-5. A. Stick model of 8-5 (shown in atom colors) superimposed onto the solid surface of thrombin (red: negative surface potential; blue: positive surface potential). Important solvent molecules are shown as red balls. Important thrombin residues are labeled. B. Stick model of the 8-5−thrombin complex. Inhibitor 8-5 is shown in pink, and thrombin in atom colors. Important thrombin residues are labeled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

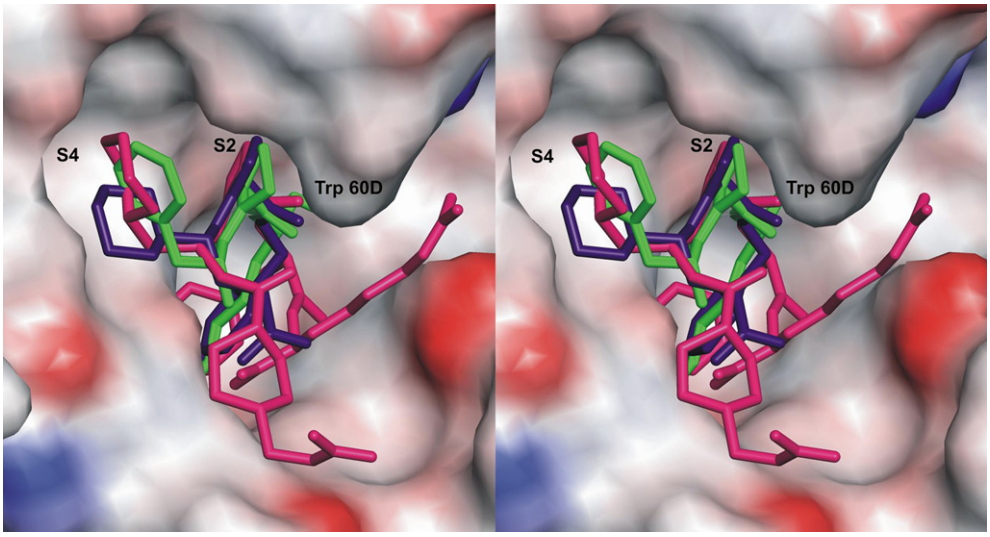

Fig. 3. Superimposition of various thrombin inhibitors. Thrombin is represented as solid surface (red: negative surface potential; blue: positive surface potential). Pink: 8-5, green: PPACK, blue: melagatran. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

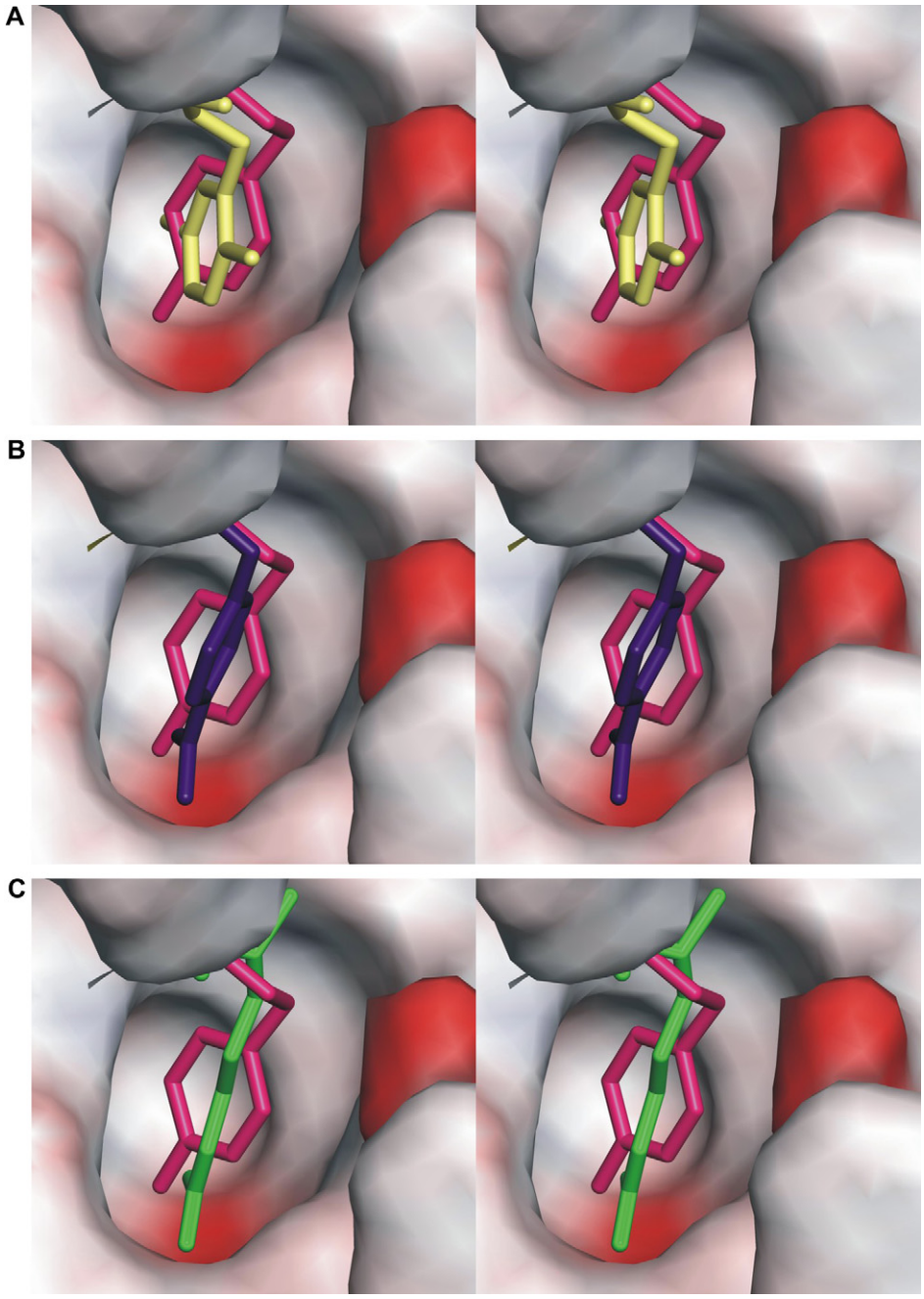

Fig. 4. Comparison of the S1 binding mode of 8-5 with other thrombin inhibitors. Thrombin is represented as solid surface (red: negative surface potential; blue: positive surface potential). A. 8-5 (pink) vs. 2,5-dichlorobenzyl derivative (yellow). B. 8-5 (pink) vs. melagatran (blue). C. 8-5 (pink) vs. PPACK (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

amidino nitrogens of melagatran (Fig. 4B) would be located. The function of the second nitrogen of a guanidino- or amidino-group is adopted by a well ordered water molecule (water 38), which bridges the second carboxyl oxygen of Asp 189 and the D-Tyr OH with distances of ~2.6 Å each. The Cβ atom of D-Tyr pushes the Ser 195 Oγ out of its normal site that is productive for catalysis ($\chi_1 = -70°$, *gauche*(+)), rotating it to a *gauche*(−) conformation ($\chi_1 = +44°$), and thus rendering it incompatible with peptide cleavage (Fig. 2B). This Ser 195 rearrangement is reminiscent of an N^{α}(Me)Arg-containing thrombin inhibitor [14], where the additional *N*-methyl group of P1-Arg is responsible for an even more unproductive conformation ($\chi_1 = +80°$) of Ser 195. The temperature (*B*) factor of the Oγ atom (33 $Å^2$) is significantly higher than that of the other Ser 195 atoms (~20 $Å^2$), indicating some mobility. In this unproductive conformation, Ser 195 Oγ can still form hydrogen bonds to the Nε2 of His 57 and to a chain of ordered water molecules (see below).

The D-configuration of the Tyr residue results in a backward folding of the inhibitor backbone, away from the course a normal all-L-amino acid substrate would take. As a consequence, the ''scissile'' peptide bond is not presented towards the

catalytic machinery, and a new hydrogen bond between the Tyr carbonyl oxygen with the backbone amide of Glu 192 (~2.7 Å) is formed, which would not be possible for an L-amino acid in this position. More importantly, the C-terminal carboxamide of the inhibitor is brought into a position where it can form two N–O hydrogen bonds with the peptide backbone of Gly 219. This interaction is protected from bulk solvent by the shielding effect of the N-terminal G-Trx cyclohexyl ring placed in front of the three novel hydrogen bonds. The side chain of the C-terminal L-homoArg (Pos. 5) perfectly slots into the narrow cleft between Trp 60D, Trp 148 and Glu 192 (Fig. 2A) and helps shielding the subjacent hydrogen bonds of the inhibitor with Glu 192 and Gly 219 from the bulk solvent. A chain of three ordered water molecules connects the guanidinium group of L-homoArg with the carboxyl group of Glu 192 and the rotated Ser 195 Oγ (Fig. 2A). These solvent molecules contribute to the extended conformation and stabilization of this well defined side chain. Surprisingly, similar to the G-Trx guanidinium group in Pos. 1, the guanidinium group of L-homoArg again does not form a direct salt bridge, this time with Glu 192, but is certainly attracted by its negative potential (Fig. 2).

The striking selectivity of 8-5 for thrombin over trypsin can be mainly explained by the different size and charge of the S4 pockets of thrombin and trypsin. On one hand, the S4 of trypsin is flat and therefore sterically not suited to accommodate D-Cha. On the other hand, trypsin possesses a Lys 175, which renders the S4 subsite more polar than the hydrophobic aryl binding site of thrombin. Moreover, the negative electrostatic potential of Glu 192 of thrombin attracts the basic homoArg side chain more than the neutral Gln 192 of trypsin.

The present study elucidates the molecular basis for the high potency and selectivity of the novel inhibitors. It also demonstrates that computer-aided molecular design [5] starting from a non-biased set of randomly chosen compounds can yield enzyme inhibitors with a novel and original binding mode. Indeed, compounds with the D-Cha-L-Aze-D-Tyr backbone represent an entirely new class of promising thrombin inhibitors with a high potential for further development.

Acknowledgements

This work was in part supported by grant BioFuture 0311852 from the Bundesministerium für Forschung und Technologie, Germany, by the European Commission as SPINE, contract no. QLG2-CT-2002-00988 under the integrated programme "Quality of Life and Management of Living Resources", and the "Fonds der Chemischen Industrie".

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