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Factor VIIa inhibitors: Target hopping in the serine protease family using X-ray structure determination

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ABSTRACT

Selective factor VIIa–tissue factor complex (FVIIa/TF) inhibition is regarded as a promising target for developing new anticoagulant drugs. Compound **1** was discovered from focused screening of serine protease-directed compounds from our internal collection. Using parallel synthesis supported by structure-based drug design, we identified peptidemimetic FVIIa/TF inhibitors (compounds **4**–**11**) containing L-Gln or L-Met as the P2 moiety. However, these compounds lacked the selectivity of other serine proteases in the coagulation cascade, especially thrombin. Further optimization of these compounds was carried out with a focus on the P4 moiety. Among the optimized compounds, **12b**–**f** showed improved selectivity.

Factor VIIa (FVIIa) is a serine protease and one of the key enzymes of blood coagulation. FVIIa in complex with tissue factor (TF) initiates the blood coagulation cascade. This complex activates factors IX to IXa and X to Xa, which activates factors X to Xa and prothrombin to thrombin, respectively.[1] Thrombin cleaves fibrinogen to fibrin, which forms blood clots with activated platelets. Inappropriate thrombus formation in blood vessels causes cardiovascular disease (myocardial infarction, stroke, pulmonary embolism, and so on), which is the most common cause of mortality in industrialized countries.[2] Therefore, the prevention of blood coagulation has become a major target for treatment of thromboembolic disorders, and much research has focused on the generation of thrombin or factor Xa selective inhibitors.[3–6] Recent studies on the inhibition of the FVIIa/TF complex have shown that selective FVIIa/TF complex inhibition may provide effective anticoagulation and low risk of bleeding compared to other antithrombotic mechanisms.[7–12] Thus, selective FVIIa/TF complex inhibition is regarded as a promising target for developing new anticoagulant drugs.[13–21]

Compound **1** was discovered from focused screening of serine protease-directed compounds from our internal collection (Fig. 1). Compound **1** showed more potent inhibition activity for thrombin than for FVIIa/TF. Using 3D-structural information of FVIIa/TF and thrombin,[22–26] we attempted to improve inhibition activity for FVIIa/TF and selectivity against thrombin. Although both FVIIa and thrombin belong to the serine protease family and their 3D structures are similar, several regions are structurally different especially the S2 site. Thrombin has an insertion loop at the S2 site, protected by hydrophobic residues Tyr60A and Trp60D. The small, hydrophobic residue proline in P2 of PPACK fits the S2 site of thrombin[25,26] and thus would also fit compound **1**. First, using solid-phase synthesis, we conducted a search for modifications of P2 which would improve inhibition activity for FVIIa/TF and selectivity against thrombin (Scheme 1).

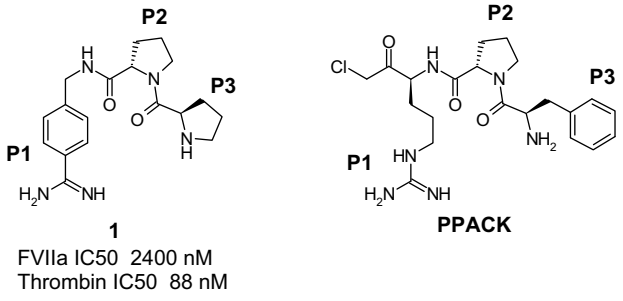

Figure 1. Structure of compound **1** and PPACK.

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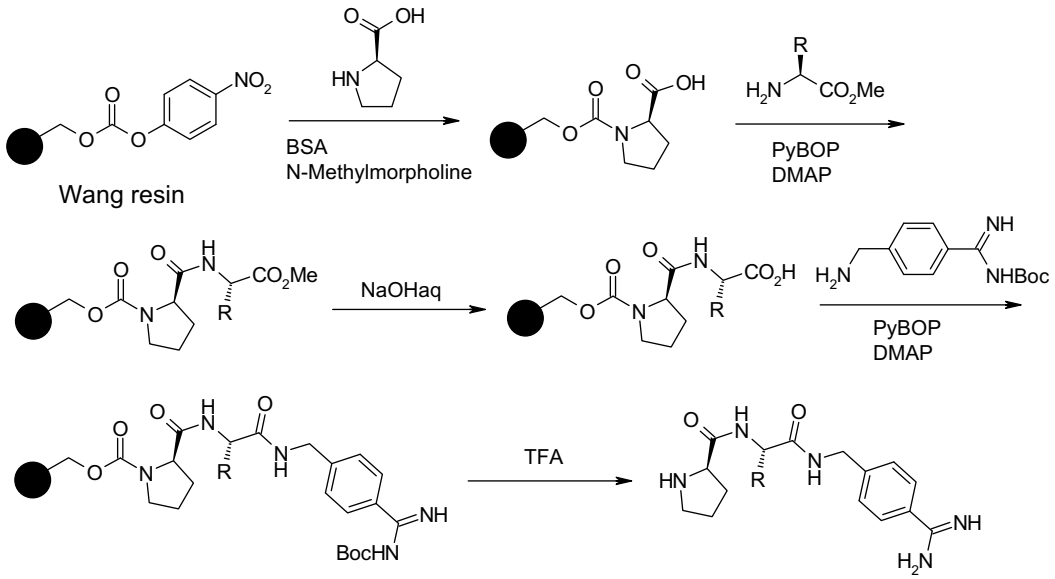


Scheme 1. Synthetic scheme of the derivatization of P2 using solid-phase synthesis.

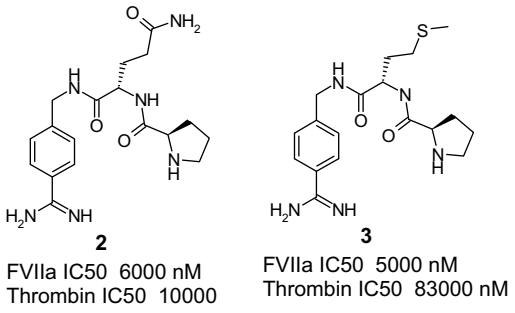


Figure 2. Structures of primary lead compounds containing L-Gln or L-Met.

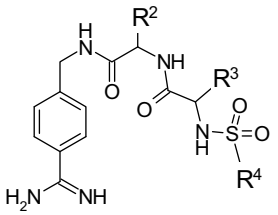
L-Gln and L-Met were considered favorable for the S2 site of FVIIa/TF but compounds **2** and **3** lacked the selectivity of the other serine proteases in the coagulation cascade and anti-FVIIa activity (Fig. 2). Therefore, we carried out further optimization focusing on P3 and P4. Here, we report the preparation and structure–activity relationships (SAR) of these compounds.[27]

First, we searched for modifications of P3 supported by structure-based drug design. We found that in reference to thrombin inhibitors,[29,30] P2 contained L-Gln or L-Met, and P4 was fixed with either a propylsulfonyl or a benzylsulfonyl group.

The S3 site of thrombin, which consists of Leu99, Ile174, and Trp215, is larger than that of FVIIa/TF because the side chain of Leu99 in thrombin extends to the S2 site whereas the side chain of Thr99 in FVIIa occludes the S3 site of FVIIa/TF.[25,26,31] Hydrophobic interactions of the D-Phe side chain in P3 of PPACK with the large S3 site of thrombin are strong and so we introduced relatively small amino acids at P3.

As a result, we generated the series of analogs depicted in Table 1. The 3D structure of compound **10** bound to FVIIa/sTF revealed that the side chain of D-Thr fits well into the small S3 site of FVIIa/sTF (Fig. 4). The small size of the P3 moiety seemed to be another key factor in the improvement of selectivity versus thrombin and the modification of P3 to D-Ile improved inhibition activity for FVIIa/TF. However, this modification caused a decrease of selectivity versus thrombin. Apparently, the D-Ile side chain in P3 formed hydrophobic interactions with the more hydrophobic thrombin S2 site, which suggested that reduction of hydrophobicity in P3 is important for the improvement of selectivity versus thrombin.

Table 1
In vitro inhibitory activities against FVIIa/TF, thrombin, and FXa[28]



Compound	R2	R3	R4	K_i (nM)			Thrombin
				FVIIa	Thrombin	Fxa	FVIIa
4	L-Gln	D-Ile	Propyl	69	500	2700	7.2
5	L-Gln	D-Ile	Benzyl	25	150	330	6.0
6	L-Gln	D-Thr	Propyl	198	13,342	NT*	67.4
7	L-Gln	D-Thr	Benzyl	114	3362	NT*	29.5
8	L-Met	D-Ile	Propyl	35	220	1500	6.3
9	L-Met	D-Ile	benzyl	15	55	160	3.7
10	L-Met	D-Thr	Propyl	130	3400	25,000	26.2
11	L-Met	D-Thr	Benzyl	42	760	1800	18.1

* NT, not tested.

The X-ray structure of compound **5** suggested that the glutamine side chain in P2 of compound **5** formed potent hydrogen bonds with Asp60, Tyr94, and Thr98 in the hydrophilic pocket of the S2 site of FVIIa/sTF (Fig. 3). However, the glutamine side chain in P2 could not form such hydrogen bonds in the S2 site of thrombin because instead of the corresponding hydrophilic pocket, thrombin has hydrophobic residues Tyr60A and Trp60D in the S2 site that well fit the small, hydrophobic proline moiety in P2 of PPACK. Therefore, the introduction of a relatively large glutamine moiety in P2 seemed to cause a steric repulsion to the residues and a reduction of inhibition activity for thrombin.

In the case of compound **10**, the X-ray structure suggested that a methionine side chain occupies a small pocket at the S2 site of FVIIa/sTF (Fig. 4), similar to other FVIIa selective inhibitors.[21] The S2 site of thrombin does not have a pocket corresponding to the pocket in the S2 site of FVIIa/sTF to accept the methionine side chain because Leu99 extends into the S2 site. So the modification of P2 from proline to a larger methionine led to a decrease in the inhibition activity of thrombin, likely caused by the unfavorable

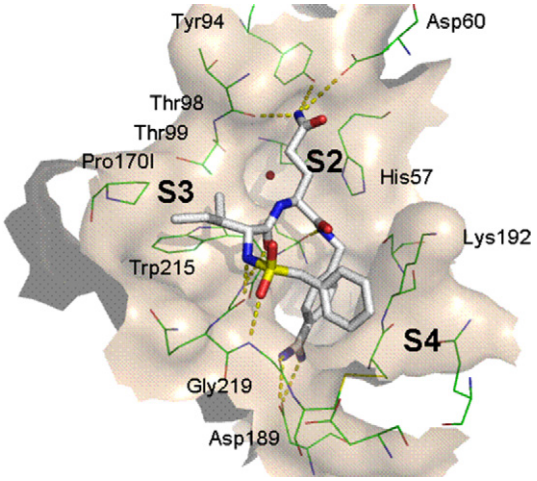


Figure 3. X-ray crystal structure of compound **5** (white) bound to FVIIa/sTF[32–34] (green). The Gln side chain at P2 creates strong hydrogen bond networks with Asp60, Tyr94, and Thr98. The relatively small S3 is a good fit into D-Ile at P3. The hydrogen bond with Gly219 and hydrophobic interactions with S4 contribute to the increase in inhibition activity.[35]

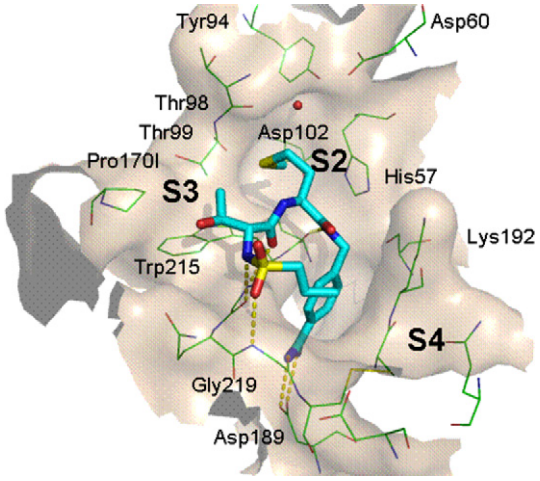


Figure 4. X-ray crystal structure of compound **10** (cyan) bound to FVIIa/sTF32-34 (green). The side chain of Met at P2 occupies a small pocket consisting of His57, Thr98, Thr99, and Asp102.[36]

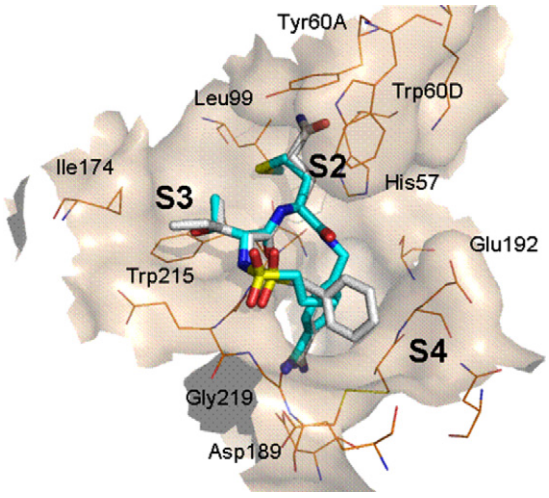


Figure 5. Superimposition of compounds **5** (white) and **10** (cyan) onto the thrombin structure (orange) in complex with PPACK[25,26] (PPACK is not shown).

steric repulsion between the large methionine side chain and the small S2 site of thrombin.

In order to improve the selectivity over thrombin, we optimized the P4 moiety based on a comparison of the crystal structure of compound **5** bound to FVIIa/sTF and the 3D-model of compound **10** bound to thrombin (Fig. 5). In both cases, the benzylsulfonamide moiety in P4 occupies the S1 subsite. Both S1 subsites are similar in size, so we focused on the difference in charge between the Lys192 of FVIIa and the Glu192 of thrombin. We hypothesized that if a carboxylic acid moiety was introduced to the benzene ring of the benzylsulfonamide moiety, the inhibition activity for FVIIa/TF would improve from a reduction of binding affinity for thrombin. The X-ray crystal structure and computational model suggested that the best position for the carboxylate group was the meta position of the benzene ring of benzylsulfonamide.

The synthesis of carboxylic acid derivatives **12a–f** is shown in Scheme 2. These compounds were synthesized using standard methods.

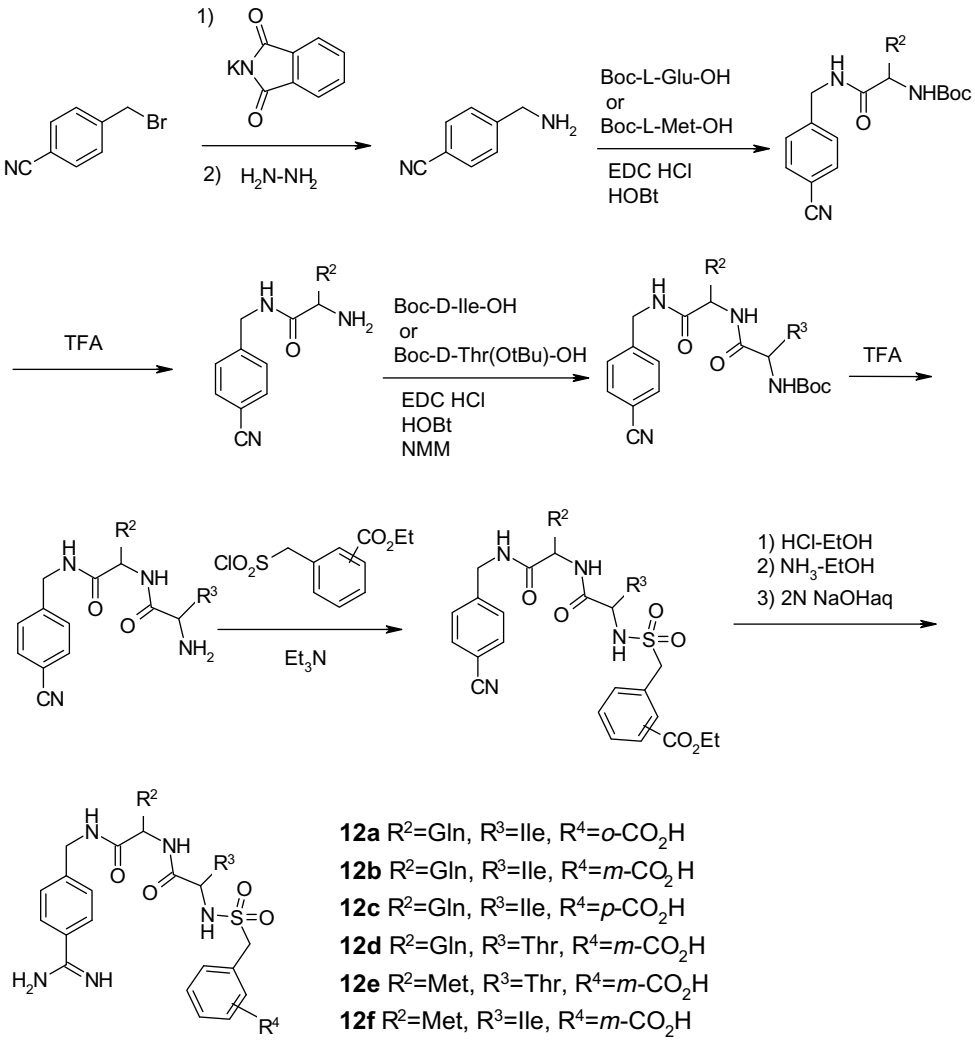
Table 2 summarizes the biological activity of the carboxylic acid analogs. As in our model, the favorable substituent position is *meta* for **12a–c**. Lead compounds **5**, **7**, **9**, and **11** led to dramatically improved selectivity of **12b**, **12d**, **12e**, and **12f** over thrombin because of the electrostatic repulsion of thrombin with Glu192. The X-ray structure of our compound with similar molecular structure suggested that the carboxylate group formed an ionic interaction with Lys192, and the O atoms of carboxylate group are 3.8 Å.[37] However, inhibition activities for FVIIa/TF of these compounds showed better retention than the lead compounds. In general, the contribution of an ionic interaction to ligand-binding free energy is not significant in a solvent-exposed region because of the free-energy penalty of the desolvation of the charged groups,[38,39] and that the side chain of Lys192 of FVIIa/TF is fully exposed to solvent. Therefore, the introduction of a charged group would not cause an increase in the inhibition activity for FVIIa/TF.

The inhibitors were also tested in standard clotting assays including a prothrombin time (PT) assay and an activated partial thromboplastin time (APTT) assay to confirm effects on blood coagulation from the improved specificity for FVIIa/TF inhibition. Theoretically, specific inhibition of FVIIa/TF, which blocks only extrinsic coagulation, should result in prolongation of only PT without affecting APTT, thus making the concentration ratio for a 2-fold prolongation of APTT and PT (2× APTT/2× PT) infinitely large. The measured 2× APTT/2× PT ratio of compound **12e** was 3.8,[40] which is higher than that of the thrombin selective inhibitor argatroban[41] (0.4, in-house data), and the FXa selective inhibitor DX-9065a[42] (2.2, in-house data).

It is generally accepted that inhibitors containing highly basic functions such as an amidine group are often poorly absorbed. So not surprisingly, the oral bioavailability of **12e** amounted to only 3.8% in rat.

A recent study on inhibitors with an amidine group has shown that when the strongly basic benzamidine group was masked as an amidoxime prodrug, oral bioavailability increased.[15] Replacement of the amidine with less basic or non-basic groups led to increased oral bioavailability.[18–20]

In summary, we succeeded in target hopping in the serine protease family using crystal structures of human FVIIa/TF in complex with peptide mimetic inhibitors. We found that L-Gln and L-Met are suitable for the S2 site of FVIIa/TF, and that the introduction of a carboxylic acid moiety to the *meta* position in benzylsulfonamide is effective for improving selectivity over thrombin. Further synthetic efforts to improve pharmacokinetics are in progress. This structural information is important in understanding the interactions that contribute to specificity for FVIIa/TF and for the rational and rapid development of specific inhibitors of FVIIa/TF.



Scheme 2. Synthetic scheme of the derivatization of P4.

Table 2
In vitro inhibitory activities against serine proteases[43]

Compound	K_i (nM)						Thrombin
	FVIIa	Thrombin	Fxa	FIXa	FXIa	FXIIa	FVIIa
12a	43	5490	3130	>100,000	113	>100,000	127.7
12b	18	5731	685	NT*	33	NT*	318.4
12c	35	680	2483	NT*	17	NT*	19.4
12d	74	45,500	14,700	NT*	259	>100,000	614.9
12e	30	25,300	5700	>100,000	323	>100,000	843.3
12f	20	3850	920	NT*	115	>100,000	192.5

* NT, not tested.

Acknowledgment

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