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Factor VIIa inhibitors: Gaining selectivity within the trypsin family

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Abstract—Within the trypsin family of coagulation proteases, obtaining highly selective inhibitors of factor VIIa has been challenging. We report a series of factor VIIa (fVIIa) inhibitors based on the 5-amidino-2-(2-hydroxy-biphenyl-3-yl)-benzimidazole (**1**) scaffold with potency for fVIIa and high selectivity against factors IIa, Xa, and trypsin. With this scaffold class, we propose that a unique hydrogen bond interaction between a hydroxyl on the distal ring of the biaryl system and the backbone carbonyl of fVIIa lysine-192 provides a basis for enhanced selectivity and potency for fVIIa.

The development of novel antithrombotic agents for the treatment of coagulation disorders is an active area of research in the pharmaceutical industry. The enzymes (Factors IIa, Xa, VIIa, IXa, and XIa) that comprise the extrinsic and intrinsic pathways of coagulation, leading to the formation of a blood clot, are trypsin-family serine proteases.[1] Preclinical models of thrombosis in several species have suggested that a selective inhibitor of the coagulation proteases earlier in the cascade (Factors VIIa and IXa) may have a greater therapeutic/safety index than inhibition of proteases later in the cascade (Factors Xa and IIa).[2–4] Based on this pharmacology guidance, we chose to develop potent and selective inhibitors of factor VIIa-tissue factor complex (fVIIa) as an effective strategy for treatment of coagulation disorders.

We have previously described the development of active site small-molecule inhibitors which interact with both fVIIa and factor Xa (fXa).[5] Within the trypsin family of coagulation proteases, developing highly selective inhibitors of Factor VIIa has proved difficult.[6] Herein, we report on the further development of our 5-amidino-2-(2-hydroxy-biphenyl-3-yl)-benzimidazole **1** scaffold to achieve increased potency for factor VIIa and high selectivity against trypsin and the late coagulation pathway proteases; fIIa, fXa (see Fig. 1).

Our efforts toward developing a selective fVIIa inhibitor began with the broad spectrum trypsin-family protease inhibitor, **1**. The potency of **1** is mediated by a unique network of hydrogen bonds to the catalytic Ser-195, common to all proteases in this family. This protease-inhibitor binding paradigm is observed at high resolution in a large set of crystal structures (>400 structures).[7–10] Compound **1** was chosen for further optimization to obtain a highly selective fVIIa compound due to its initial potency for fVIIa (K_i = 0.074 μM), high solubility, and excellent parenteral pharmacokinetic profile.[11]

	K_i(μM)
fVIIa	0.074
fIIa	210
fXa	2.4
Trypsin	6.7

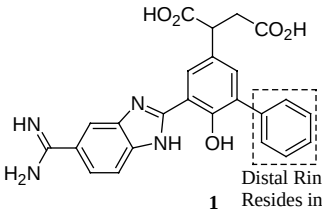

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Figure 1. The potency for **1** versus fVIIa, fXa, fIIa, and trypsin.

Keywords: Factor VIIa; Trypsin; Factor Xa; Thrombin; fIIa; Selectivity; Suzuki; Amidine; Lysine-192; Crystallography; Inhibitor.
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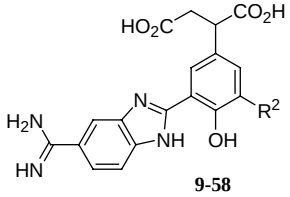
Compound **1**, while possessing good selectivity for IIa, had suboptimal selectivity for fXa and trypsin. Our initial goal was to gain 1000-fold selectivity for fVIIa versus fIIa, fXa, and trypsin (Table 1). From crystallography and modeling analysis, further improvements in selectivity against fXa and trypsin were envi-

Table 1. SAR data for selected compounds **1**, **9**–**58**



9-58

Compound	R^2	Selectivity ratios versus fVIIa			
		fVIIa K_i (μM)	IIa	Xa	Trypsin
1	Phenyl	0.074	2,838	32	91
9	**2-Hydroxy-5-fluorophenyl**	**0.004**	**>55,500**	**1800**	**4400**
10	**2-Hydroxy-5-chlorophenyl**	**0.0054**	**27,800**	**722**	**2500**
11	**2-Hydroxy-5-nitrophenyl**	**0.006**	**25,000**	**1450**	**8500**
12	**2-Hydroxy-5-aminophenyl**	**0.010**	**15,800**	**505**	**1789**
13	**2-Hydroxy-5-cyanophenyl**	**0.012**	**12,000**	**848**	**2960**
14	**2-Hydroxyphenyl**	**0.013**	**20,800**	**458**	**1077**
15	2-Hydroxy-3-bromo-5-chlorophenyl	0.009	17,000	216	398
16	2-Hydroxy-3,5-dichlorophenyl	0.014	10,700	171	421
17	2-Hydroxy-4,6-dichlorophenyl	0.025	6000	112	192
18	3-(Hydroxymethyl)phenyl	0.021	5744	86	216
19	3-Nitrophenyl	0.022	9545	42	268
20	2-Nitrophenyl	0.022	5455	109	25
21	3,5-Dichlorophenyl	0.027	33,300	43	98
22	3,5-Dimethylphenyl	0.029	29,700	62	1077
23	3-Acetylphenyl	0.033	5512	73	197
24	3-Aminophenyl	0.036	3333	128	250
25	3-Methylphenyl	0.038	3947	61	108
26	*N*-(3-Methylphenyl)acetamide	0.054	2778	28	102
27	2-Thiomethylphenyll	0.064	1719	63	103
28	3-Chlorophenyl	0.066	2273	24	59
29	3,5-Difluorophenyl	0.068	9706	24	115
30	3-Isopropylphenyl	0.076	11,800	17	40
31	3-Cyanophenyl	0.077	1458	21	71
32	3-Hydroxyphenyl	0.088	1705	41	99
33	5-Chlorothiophene	0.11	5636	17	10
34	3-Acetamidylphenyl	0.11	1182	51	118
35	3-(Difluoromethoxy)phenyl	0.12	5917	16	51
36	2-Methoxyphenyl	0.12	3750	30	55
37	3-Chloro-4-fluorophenyl	0.13	6923	15	34
38	2-Methoxyphenyl	0.13	1154	54	123
39	5-(Hydroxymethyl)thiophene	0.13	1100	25	33
40	2-Fluorophenyl	0.135	1000	17	43
41	2,3,5-Trichlorophenyl	0.21	714	35	39
42	2,5-Dichlorophenyl	0.25	600	20	35
43	2,3-Dichlorophenyl	0.27	556	31	44
44	3,4-Phenyldioxolone	0.28	536	13	20
45	2-Methoxy-5-cyanophenyl	0.28	540	24	54
46	2-Methoxy-5-fluorophenyl	0.33	455	39	52
47	2-Aminophenyl	0.42	357	18	41
48	4-Methylphenyl	0.42	310	11	8
49	4-Chlorophenyl	0.44	341	7	2
50	2-Methylphenyl	0.50	200	12	16
51	3-Pyridyl	0.55	209	14	31
52	2-(Hydroxymethyl)phenyl	0.73	205	13	16
53	3-(Aminomethyl)phenyl	0.78	192	9	18
54	4-Hydroxyphenyl	0.88	170	14	10
55	4-Methoxyphenyl	2.25	67	3	1
56	2-Acetylphenyl	4.0	38	16	28
57	H	6.4	>24	3	1
58	4-*tert*-Butylphenyl	16	9	4	1

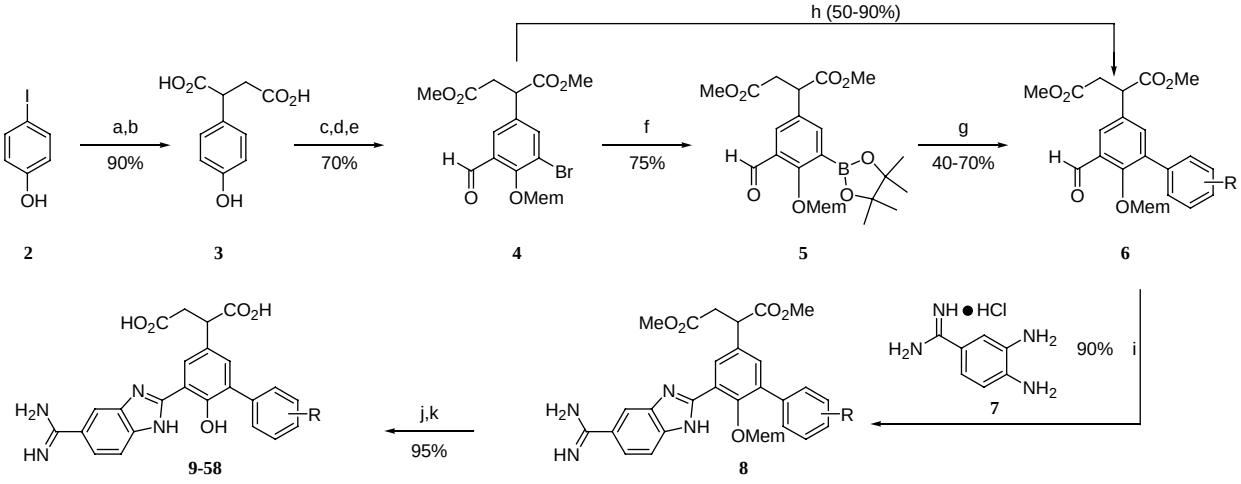
Data shown are factor VIIa K_i and *fold*-selective ratios (anti-target K_i/fVIIa K_i) for coagulation factors IIa, Xa, and trypsin.[16,17]

sioned via modifications of the distal biaryl ring. The distal aryl ring of **1** resides within the S1′-pocket of trypsin-family proteases. Due to the structural variation amongst the trypsin-family proteases in the S1′-pocket, we chose to gain further selectivity for fVIIa by exploring this region. This strategy was executed by generating a varied set of distal substituted biaryl compounds via a Suzuki-based synthesis strategy.

Synthesis of the distal-arene-substituted analogs of **1** began via a Heck coupling of dimethyl fumarate with commercially available 4-iodophenol **2** (Scheme 1). The subsequent olefin was reduced with hydrogen and Pearlman's catalyst, followed by selective *ortho*-formylation of **3** with paraformaldehyde and $MgCl_2$ using the conditions of Hofslokken et al.[12] The resulting salicylaldehyde was brominated with *N*-bromosuccinimide in DMF, followed by protection of the phenol with MemCl to afford aryl ether **4**. The distal-arene diversity was installed by a Suzuki mediated coupling of aryl bromide **4** with selected boronic acids (Scheme 1, reaction h). Alternatively, when the corresponding boronic acid was not readily available, the aryl bromide **4** was converted to the boronic acid pinacol ester **5** by reaction of **4** with bis(pinicolato)diboron and $PdCl_2$(dppf) as a catalyst.[13] (Scheme 1, reactions f and g) The benzimidazole ring system was constructed via a 1,4-benzoquinone mediated oxidative cyclization of the aryl aldehyde **6** with 3,4-diaminobenzamidine hydrochloride **7**. To provide the desired fVIIa inhibitor, the phenol was deprotected with methanolic HCl, followed by treatment with refluxing 1 N aqueous HCl to hydrolyze the methyl esters to the corresponding carboxylic acids. Utilizing this synthetic strategy, compounds **1** and **9**–**58** were obtained. All compounds were purified by preparative reverse-phase HPLC, and isolated as their corresponding HCl salts after lyophilization. All compounds were synthesized and characterized in the inhibition assays as a racemic mixture.

From the set of 50 compounds generated via this Suzuki-based synthesis strategy, fVIIa potencies ranging from 4 nM (**9**) to 16 μM (**58**) and varying selectivities were observed. This range of activity illustrated that simple substitutions on the distal ring of **1** can effectively sample the structural variations within the S1′-pocket. Overall, this initial strategy generated 21 compounds more potent than the initial unsubstituted base scaffold **1** (Table 1). Nevertheless, the majority of the compounds surveyed within this set had comparable selectivity to **1**, with Factor Xa and trypsin selectivities below our set criteria. However, compounds **9**–**14**, containing the *ortho*-phenol on the distal aryl ring ($\mathbf{R}^2$, Table 1), demonstrated significantly improved selectivity and potency over all other compounds within this diversity set. The simple conversion of the 2-aryl hydrogen of base scaffold **1**, to a 2-hydroxy (compound **14**), provides a 5-fold increase in potency and a 10-fold increase in selectivity. Increasing the acidity of this distal phenol by the incorporation of a fluorine *para* to the phenol provided another 5-fold increase in potency, resulting in the fVIIa inhibitor **9** (fVIIa K_i = 4 nM), which significantly surpassed our initial selectivity criteria and yielded a selectivity of >55,500-fold against fIIa, 1800-fold selectivity against fXa and 4400-fold selectivity against trypsin. To highlight the importance of the 2-hydroxy for selectively targeting fVIIa, capping of the hydroxy in **9** as the corresponding methyl ether **46** resulted in a 100-fold loss in potency. Additional substitutions to the 2-hydroxy distal aryl ring, at sites other than the 5-position, caused fXa and trypsin selectivity to decrease (**15**–**17**).

In an effort to explain the enhanced selectivity and potency derived from the distal 2-hyroxyphenyl on compounds **9**–**14**, we developed a hypothesis based on a unique fVIIa structural change observed by Banner and coworkers in fVIIa/sTF X-ray structures.[14] In this report, Banner describes a unique Lys-192, Gly-193 main



Scheme 1. Reagents and conditions: (a) $Pd(OAc)_2$, (*o*-Tol)$_3$P, TEA, dimethyl fumarate; (b) MeOH, 50 psi H_2, $Pd(OH)_2$ on carbon (Pearlman's catalyst); (c) $MgCl_2$, anhyd paraformaldehyde, TEA, CH_3CN, reflux, 4 h; (d) NBS, DMF; 0 °C (e) 2-methoxyethoxymethyl chloride (MemCl), TEA, CH_2Cl_2; (f) bis(pinicolato)diborane, $PdCl_2$(dppf), KOAc, DMF; (g) $Pd(PPh_3)_4$, aq K_2CO_3, DME, MeOH, ArBr or ArI, 85 °C; (h) $Pd(PPh_3)_4$, aq K_2CO_3, DME, MeOH, $ArB(OH)_2$; (i) 3,4-diaminobenzamidine·HCl, 1,4-benzoquinone (1 equiv), MeOH, reflux 6 h; (j) 4 N HCl in dioxane/MeOH 1 h; (k) aq 1 N HCl reflux, 1 h.

chain amide which is capable of a 180°-rotation due to a hydrogen bond to Gln-143. This rotation repositions the Lys-192 main chain carbonyl into the oxy-anion hole, removing one of the essential catalytic features of the active site. This main chain amide rotation is not likely in the other human trypsin-family proteases since they do not have an equivalent residue corresponding to the Gln-143 of fVIIa. This unique fVIIa rearrangement provides the basis for a potential fVIIa selective interaction. When compound **9** is modeled in the active site of fVIIa, the distal 2-hydroxyphenyl is in a favorable orientation to form a hydrogen bond between the rotated main chain carbonyl of Lys-192 and the hydrogen of the phenol.[15] This potential hydrogen bond we hypothesize is the basis for our observed increase in potency and selectivity with compounds **9**–**14**.

In conclusion, a systematic SAR study on the distal ring of **1** revealed that the scaffolds which incorporate a 2-hydroxy on the terminal aryl ring display significantly enhanced potency and selectivity. Accordingly, these analogs are candidates for further development and the results of such work will be disclosed in due time.

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16. General Inhibition Assays. Inhibitor potency measurements were performed at room temperature using either a Molecular Device's SpectraMax 250 (absorbance assays) or fMax (fluorescence assays) 96-well kinetic plate readers. Reaction velocities were monitored at varying inhibitor concentrations by following the hydrolysis of either *para*-nitroanalide (A405 nm) or aminomethylcoumarin substrates (ex355, em460). All substrates were added at concentrations equal to or near their K_m. All reactions were performed in a total volume of 100 μL. Control reactions in the absence of inhibitor were performed in parallel. Factor VIIa: Factor VII (Enzyme Research) was incubated at 7 nM together with recombinant soluble human tissue factor (11 nM) with variable concentrations of inhibitor in 50 mM Tris (pH 7.4), 150 mM NaCl, 5.0 mM $CaCl_2$, 1.5 mM EDTA, 0.05% Tween 20, and 10% DMSO. The reaction was initiated with the addition of substrate, CH_3SO_2-D-CHA-But-Arg-pNA (Centerchem), supplied at the K_m (500 μM). The change in absorbance as a function of time was monitored at 405 nm. Factor IIa (Thrombin). Thrombin (Calbiochem) was incubated at 12.7 nM with variable concentrations of inhibitor in 50 mM Tris (pH 7.4), 150 mM NaCl, 5.0 mM $CaCl_2$, 1 mM EDTA, 0.05% Tween 20, and 10% DMSO. The reaction was initiated with the addition of substrate, Tosyl-Gly-Pro-Lys-pNA (Centerchem), supplied at the K_m (25 μM). The change in absorbance as a function of time was monitored at 405 nm. Factor Xa. Factor Xa (Haematologic Technologies) was incubated at 2 nM with variable concentrations of inhibitor in 50 mM Tris (pH 7.4), 150 mM NaCl, 5.0 mM $CaCl_2$, 1.5 mM EDTA, 0.05% Tween 20, and 10% DMSO. The reaction was initiated with the addition of substrate, CH_3OCO-D-Cha-Gly-Arg-pNA (Centerchem), supplied at the K_m (1.0 mM). The change in absorbance as a function of time was monitored at 405 nm. Trypsin. Trypsin (Athens Research Institute)

was incubated at 10 nM with variable concentrations of inhibitor in 50 mM Tris (pH 7.4), 150 mM NaCl, 1.5 mM EDTA, 0.05% Tween 20, and 10% DMSO. The reaction was initiated with substrate, Tosyl-Gly-Pro-Lys-pNA (Centerchem), supplied at the K_m (25 μM). The change in absorbance as a function of time was monitored at 405 nm.

17. K_i apparent (K_i') values were determined by a non-linear least-squares regression fit of the experimentally derived data to the Morrison equation for tight-binding inhibitors as described (Kuzmic, P. et al., *Anal Biochem*, **2000**, *281*(1), 62). Enzyme and inhibitor were incubated 30-min prior to initiation of reaction with the addition of substrate.