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Discovery of novel hydroxy pyrazole based factor IXa inhibitor

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Abstract—Synthesis and biological data of a novel selective and efficacious factor IXa inhibitor are described along with its crystal structure in factor VIIa.

Factor IXa (fIXa) plays a key role in maintaining internal homeostasis in the intrinsic pathway of the clotting cascade. Inhibition of fIXa presents an alternative and viable way of treating thrombosis arising from both venous as well as arterial vascular injuries. It is believed that inhibiting fIXa selectively limits thrombosis in low tissue factor sites, but does not inhibit clotting in high tissue factor environments such as vascular injury sites or surgical wounds.[1] This method could provide a choice of anti-coagulants with improved therapeutic index compared to existing therapies which target thrombin.[1]

We realized that in order to develop fIXa inhibitors for various surgical indications, it is important to have selective inhibitors. The compounds need to be selective against various trypsin-like serine proteases and, more importantly, should be able to distinguish between intrinsic and extrinsic coagulation pathways. In other words, compounds should exhibit minimal efficacy in a 2× PT assay (measure of effect on the extrinsic pathway) but should have good efficacy in a 2× APTT assay[2] (measure of effect on the intrinsic pathway). In this communication, we present our results on the discovery of a novel triaryl scaffold for selective inhibition of fIXa.

As a part of our ongoing quest for novel anti-coagulants for the treatment of various cardiovascular disorders,[3] we screened our proprietary small-molecule libraries for activity against fIXa. The 5-amidino-benzimidazole analog **1** was a reasonable lead with a 99 nM potency for fIXa, however, this potency did not translate into observed efficacy in ex vivo clotting efficacy assays (2× PT and 2× APTT >10 μM). In addition, although, **1** has good thrombin selectivity it lacked selectivity against factors Xa and VIIa, and therefore warranted further optimization.

We were aware from our previous work in this area that the physicochemical properties of compounds can impact their ex vivo efficacy,[3] with increased hydrophilicity (C Log *P*) of compounds resulting in improved ex vivo efficacy. In this connection, we were interested in isosteric replacement of the central phenol in compound **1** with a polar heterocycle, which avoids significantly increasing the molecular weight. Among numerous analogs synthesized, we found the hydroxy pyrazole compound **2** to be a promising lead (Fig. 1).

Replacing the central six-membered ring with a five-membered ring, as in **2**, would be expected to bring about a change in the way the distal phenyl group extends from the scaffold due to a geometry difference. In addition, there might be a difference in the ability of the hydroxyl moiety to hydrogen bond to the protein due to changes in electronics. Gratifyingly, replacing the central phenol with a hydroxy pyrazole not only improved potency against fIXa[4] but also had a positive effect on efficacy (2× APTT = 2.6 μM). It is likely that replacement of the phenol with the more polar heterocycle led to improved physicochemical properties which in turn translated into better ex vivo efficacy. Compound **2** has improved selectivity against the other coagulation enzymes—thrombin, factor Xa (fXa), and factor VIIa

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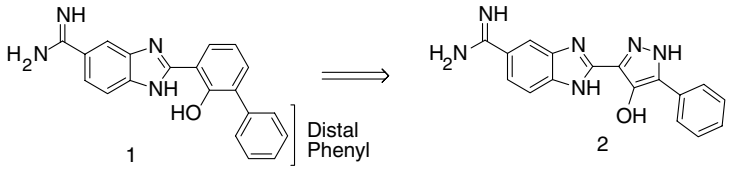


Figure 1. Isosteric replacement of the central phenol with hydroxyl pyrazole.

(fVIIa).[4] Selectivity toward tryspin was slightly diminished. This compound had low efficacy in the 2× PT assay implying that only intrinsic and not extrinsic pathway is affected, presumably due to selective fIXa inhibition.

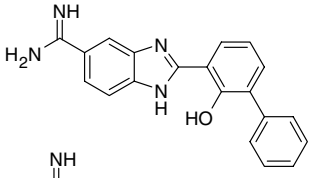
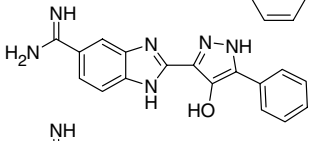
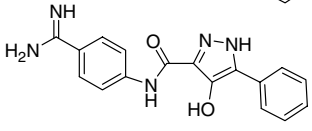
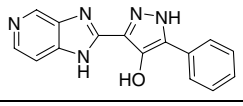
Next we were interested in finding out if the benzimidazole in compound **2** is necessary for its potency toward serine proteases. Toward this end, compound **3**, which is a simple amide linked to a hydroxy pyrazole, was made and tested. Not surprisingly, it did not have any activity against serine proteases possibly due to disruption of the well coordinated multi-centered hydrogen-bonding interactions responsible for potency against serine proteases.[5] In the absence of the amidine, as in compound **4**, there is complete lack of potency for any of the serine proteases due to loss of key interaction with Asp 189 in the S1 pocket. In compounds such as **1** and **2**, the amidine, in combination with the imidazole NH and 2′-phenol OH, is integral to the binding of this class of inhibitors to trypsin-like enzymes (Table 1).[5]

As a first step to gain an understanding of the factors controlling the potency and selectivity displayed by **2**, a crystal structure in fVIIa was obtained (Fig. 2).[6] Despite the change from a phenol to a hydroxy pyrazole in the center of the molecule, **2** binds very similarly to other molecules of this type in fVIIa.[3] A similar array of hydrogen bonds including short H-bonding interactions between Ser195, the hydroxy pyrazole oxygen, and a water molecule in the oxyanion hole was observed and the interaction of the amidine with Asp 189 was also comparable. The major differences are likely attributable to the 142° versus 120–125° angle between the P1 amidino-benzimidazole and the distal phenyl ring imparted by the five-membered ring of **2**. The distal phenyl ring of **2** angles away from the surface of the S1′ pocket which is not the case for central phenol ring containing analogs. This change may in turn be responsible for the observed closer approach of the hydroxyl of **2** to Ser195.

Comparison of the crystal structure of **2** in fVIIa to the literature structure of 4-amino-benzamidine in human fIXa[7] reveals some key differences that may be responsible in part for the selectivity. The position of the Asp189 in fIXa is more than 0.75 Å lower in the S1 pocket than for fVIIa and similar to the position observed for Asp189 of u-PA.[8] As a result, the amidine of the ligand in the literature fIXa structure is nearly a full angstrom deeper in the S1 pocket than the amidine of **2**. A second difference between the fVIIa structure of **2** and that of fIXa is the position of the 30s loop due to an insertion in fIXa. The C-α of Phe41 in fIXa is shifted 2.8 Å closer to the S1 pocket than that of residue 41 (C-α) in fVIIa, and this alters the S1′ pocket shape. The Phe41 side chain is thus located much closer to the distal phenyl ring of **2**.

Both of these structural differences, in concert with the opened angle of the central ring of **2**, may help rationalize the observed selectivity. The more linear nature of **2**

Table 1. SAR comparing **1** with hydroxyl pyrazole analogs[4]

Compound	Structure	fIXa K_i (μM)	2× APTT (μM)	2× PT (μM)	C Log *P*	fIXa Selectivity against			
						Thrombin	fVIIa	fXa	Trypsin
1		0.099	>10	≫10	3.89	247	9	32	88
2		0.05	2.64	14.5	2.61	813	36	96	44
3		120	—	—	2.32	1	1	1	—
4		110	—	—	2.72	0.72	1	1	—

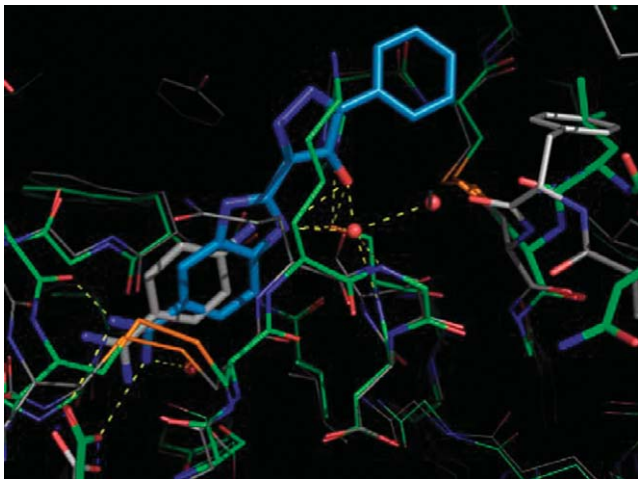
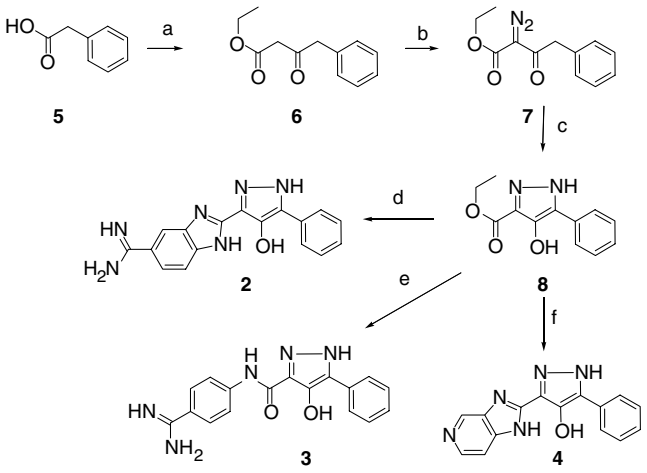


Figure 2. X-ray crystal structure of **2** in fVIIa (green) superimposed onto human fIXa with 4-amino-benzamidine (PDB entry code 1rfn:grey). Residues 57, 102, 191–194, 213–216, and 225–228 (rms = 0.33 Å) were used to overlay the proteins.



Scheme 1. Synthesis of pyrazole based compounds **2**–**4**. Reagents and conditions: (a) i—$(COCl)_2$, CH_2Cl_2; ii—Meldrum's acid, pyridine, EtOH, 0 °C to rt, overnight; (b) *p*-TsN_3, NEt_3, CH_3CN, 15 °C, 1 h; (c) NaH, THF, 0 °C to rt, 24 h; (d) 3,4-diamino-benzamidine, PPA, 140 °C, 4 h; (e) 4-amino-benzamidine, DMF heat; (f) pyridine-3,4-diamine, PPA, 140 °C 4 h.

and the resulting unique orientation of the distal phenyl ring described above, as compared to central phenol ring containing molecules, may allow it to bind further down in the S1 pocket to match the deeper pocket of fIXa. The fact that the distal phenyl ring is pulled away from the S1′ pocket may also be beneficial for fIXa binding due to the shift in the position of the 40s loop. Thus, the side chain of Phe41 may actually be in a better position for packing against the distal phenyl ring.

Phenyl acetic acid **5** was converted to β-ketoester **6** via a reported procedure.[9] β-ketoester **6** was treated with 4-carbamoyl-benzenesulfonyl azide and a base to get the diazo derivative **7**. Compound **7** upon treatment with sodium hydride[10] furnished the requisite pyrazole ester **8**. Heating the ester **8** with either diamino-benzamidine[11] or pyridine diamine in polyphosphoric acid (PPA) yielded the corresponding benzimidazole derivatives **2** and **4**. Treatment of ester **8** with *p*-amino-benzamidine at 100 °C for 2 h afforded the necessary amide **3**. Synthesis of compound **1** was reported earlier.[12]

By replacing the central six-membered phenol of **1** with a hydroxy pyrazole, we were successful in producing a novel triaryl scaffold which selectively and efficaciously inhibits fIXa. The fVIIa crystal structure with compound **2** was obtained and compared with a published structure of fIXa. This revealed some key structural features which may be responsible for the observed selectivity seen for fIXa. In addition, the presence of the heterocycle in the core of the structure possibly helped improve its physicochemical properties, which is reflected in an ex vivo clotting assay (Scheme 1).

Acknowledgments

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4. Inhibition assays for factor Xa and thrombin were performed as described (Cregar, L.; Elrod, K. C.; Putnam, D.; Moore, W. R. *Arch. Biochem. Biophys.* **1999**, *366*, 125) with the pH adjusted to 7.4. The fVIIa trypsin and fIXa assays were performed and analyzed as in the above reference with the following additional details. Factor VIIa (Enzyme Research) was incubated at 7 nM and CH_3SO_2-D-CHA-But-Arg-pNA (Centerchem) was used as the substrate. The buffer for the factor VIIa assay was supplemented with 11 nM relipidated tissue factor and 5 mM $CaCl_2$. 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). Factor IXa (Haematologic Technologies) was incubated at 16.6 nM with variable concentrations of inhibitor in 50 mM Tris (pH 7.4), 100 mM NaCl, 5.0 mM $CaCl_2$,

1.0 mM EDTA, 30% ethylene glycol, 0.05% BSA, and 10% DMSO. The reaction was initiated with substrate CH_3SO_2-D-CHG-Gly-Arg-AMC (Pentapharm) supplied at the K_m (100 μM). The change in fluorescence as a function of time was monitored (ex_{355} em_{460}).

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