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D-Phenylglycinol-derived non-covalent factor Xa inhibitors: Effect of non-peptidic S4 linkage elements on affinity and anticoagulant activity

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Abstract—Analogs to a series of D-phenylglycinamide-derived factor Xa inhibitors were discovered. It was found that the S4 amide linkage can be replaced with an ether linkage to reduce the peptide character of the molecules and that this substitution leads to an increase in binding affinity that is not predicted based on modeling. Inhibitors which incorporate ether, amino, or alkyl S4 linkage motifs exhibit similar levels of binding affinity and also demonstrate potent in vitro functional activity, however, binding affinity in this series is strongly dependent on the nature of the S1 binding element.

The need for a new oral therapy for the prevention of thrombosis has driven more than a decade of research into inhibition of the trypsin-like serine proteases involved in the coagulation cascade.[1] Reports of phase III clinical trial efficacy of ximelegatran, an oral direct thrombin inhibitor, for prevention of venous thromboembolism highlight the potential value of this overall approach even though ximelegatran was ultimately withdrawn due to adverse liver effects.[2] Amplification during the coagulation cascade suggests that a strategy of inhibition of coagulation factors that operate earlier in the cascade sequence may result in lower concentrations of inhibitor necessary to achieve efficacy and may thus lead to therapeutic agents with potential for fewer non-target related side effects. Factor Xa, a serine protease centrally located at the junction of the intrinsic and extrinsic pathways, forms a prothrombinase complex which is directly responsible for the activation of prothrombin to thrombin. It has been reported that one molecule of factor Xa in the prothrombinase complex can activate 138 molecules of thrombin per minute.[3] As such, inhibition of factor Xa has been specifically targeted by many industrial and academic laboratories.[4]

As part of our research program aimed at the discovery of selective non-covalent factor Xa inhibitors, we evaluated a series of D-phenylglycinamide-derived inhibitors having a secondary amide linkage connecting the S4 binding element to the core of the scaffold represented by structure **1** (Fig. 1).[5]

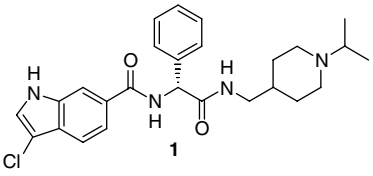

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Figure 1. A D-phenylglycinamide-derived inhibitor of factor Xa.

Keywords: Factor Xa; Serine protease inhibitors; Non-covalent inhibitors; Coagulation cascade.
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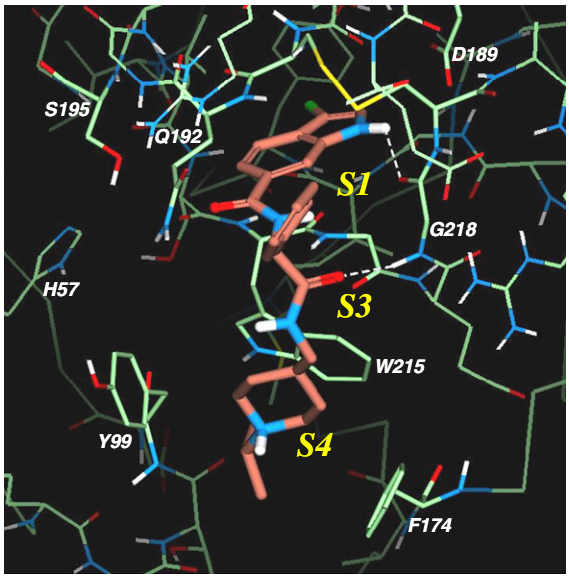
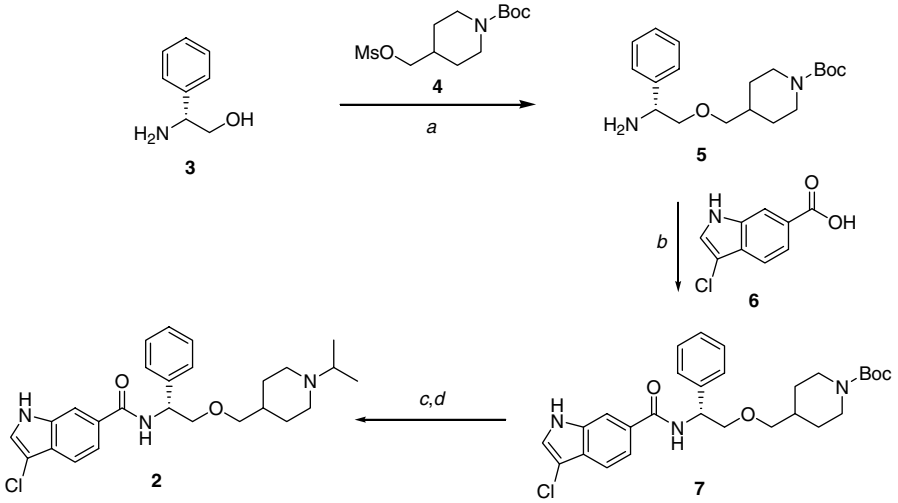


Figure 2. Computationally derived binding model of factor Xa inhibitor **1** having a secondary amide S4 linker.

Computational modeling based on X-ray co-crystal structures of related molecules can be used to develop a binding mode for structure **1** in the factor Xa active site (Fig. 2).[6] Using this approach, the chloroindolyl moiety fits into the S1 pocket such that the indole nitrogen lies between a carboxyl oxygen of Asp189 and the carbonyl oxygen of Gly218 to which it forms a hydrogen bond. This arrangement positions the chlorine near the face of Tyr228 which is located in the back of the pocket. Projecting out of the S1 pocket is the phenyl glycine-derived central portion of the inhibitor which places the edge of the phenyl ring within proximity to interact with the disulfide bridge formed by Cys191 and Cys220. The cationic box of the S4 pocket, defined by Tyr99, Trp215, and Phe174, is then filled by the *N*-isopropyl piperidinyl tail section of the inhibitor. Interestingly, the amide carbonyl which serves as the link to the S4 binder is modeled to be within hydrogen bonding distance of Gly218. Compounds were discovered that inform the model with respect to the role of the proposed amide hydrogen bond between the S4 amide linker and the protein backbone. The corresponding ether analog **2** can be prepared via alkylation of D-phenyl glycinol **3** with *N*-boc-4-methanesulfonyloxymethylpiperidine **4** to provide **5** in 52% yield. Amide coupling with 3-chloroindole-6-carboxylate **6** provides intermediate **7** which can be carried on without purification. Standard deprotection of the *tert*-butoxycarbonyl protecting group followed by reductive amination with acetone provides **2** in straightforward fashion (Scheme 1).[7]

Surprisingly, **2** demonstrates an improvement in binding affinity for factor Xa relative to compound **1**, and has an apparent association constant (K_{ass}) of 1700×10^6 L/mol compared with a K_{ass} of 460×10^6 L/mol for **1**.[8] Removal of the potential hydrogen bonding interaction predicted by modeling does not diminish activity and an X-ray co-crystal structure of **2** bound in the active site of factor Xa helps to explain the key interactions (Fig. 3).

Analysis of the factor Xa co-crystal structure with **2** shows the inhibitor binding in a very similar fashion as the model for **1** (Fig. 2). Without an amide S4 linkage, no hydrogen bond exists between the inhibitor and backbone amide nitrogen of Gly218. However, in its place, a crystallographically resolved water molecule appears to have the capability of creating a hydrogen bonding network between the ether linkage and the protein backbone amide nitrogen of Gly213. Structurally it is unclear why **2** is more active than **1**, but this result suggests that there may be differences in the binding mode of **1** that the model has not adequately captured. It is possible that there is more strain energy associated with the binding mode derived for **1** compared to that observed in the crystal structure for **2**. Another explanation may be that the unassociated **1** may exist in solution in a peptide-like C7 conformation having a well-defined intramolecular hydrogen bond. This internal interaction would need to be broken before **1** could adopt the



Scheme 1. General synthesis of S4-ether linked factor Xa inhibitors illustrated by the preparation of **2**. Reagents and condition: (a) **4**, NaH, THF:CH_3CN (5:1), 65 °C, 52%; (b) **6**, EDCl, HOAt, DMF; (c) 4 N HCl in dioxane; (d) acetone, $NaCNBH_3$, HOAc, MeOH, 54% (three steps).

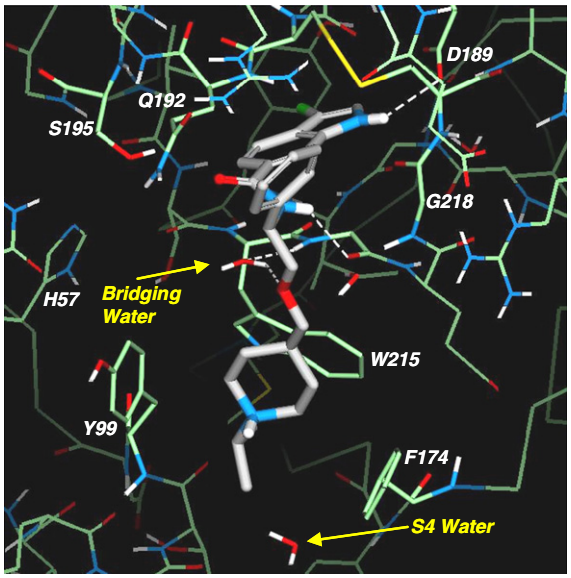


Figure 3. X-ray co-crystal structure of inhibitor **2** bound in factor Xa (1.9 Å resolution).

bound conformation modeled in Figure. 2. No such hydrogen bond is possible for **2**, so there may be less of an energy penalty to assuming the observed binding mode (Fig. 3).

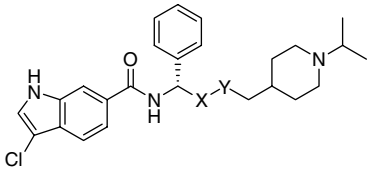
The preparation of analog **11**, which replaces the ether in **2** with an alkyl linkage, is shown below (Scheme 2). Readily available **8** can be subjected to sequential oxime formation, reduction, and boc-protection, to provide **9** in good overall yield. Reduction of the pyridine ring using platinum oxide followed by reductive amination with acetone and sodium cyanoborohydride provides intermediate amine **10**. Boc-deprotection of **10** followed by coupling with **6** provides the racemic material **11** in 20% yield (unoptimized over four steps).[7]

The all carbon linker, which is not able to engage in a bridged hydrogen bond with water, demonstrates binding affinity similar to that found for ether linked **2** (Table 1). Additional analogs containing an amino functionality in the linker can be prepared in straightforward fashion from D-phenyl glycine to provide additional information. Secondary amine containing **12** also demonstrates similar affinity to **2** and **11**, but the N-methylated analog **13** is much less active in this assay.

Additional inhibitors containing S4 ether linkers show that the 3-chloroindolyl S1 binder and the centrally located phenyl ring are key to maintaining strong affinity for the enzyme (Table 2). Interestingly, *ortho*-chloro substitution on the central phenyl ring does not provide an increase in affinity as has been reported in related systems containing a secondary amide S4 linkage.[5] Isopropyl and *n*-butyl replacements for the phenyl ring afford inhibitors **19** and **20** with markedly decreased affinity for factor Xa further highlighting the importance of the phenyl's interaction with the disulfide bridge.

Variably sized substituents on the piperidine S4 binder demonstrate interesting SAR trends (Table 3). While

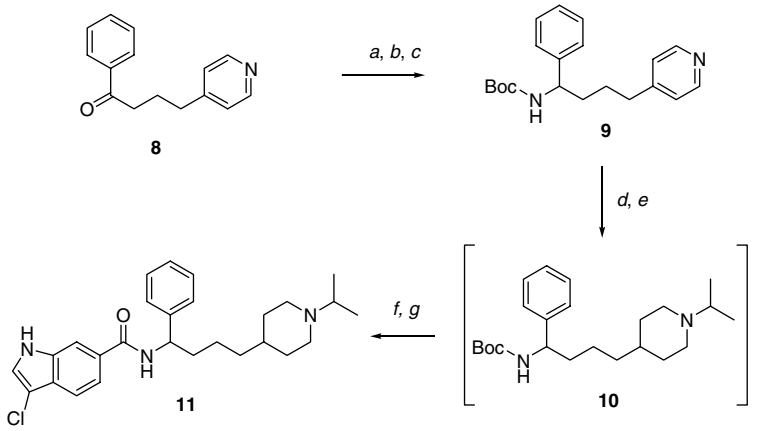
Table 1. Effect of linker on binding to factor Xa



Compound	X	Y	K_{ass}[a] (10^6 L/mol)
2	CH_2	O	1700
11	CH_2	CH_2	700[b]
12	CH_2	NH	1200
13	CH_2	NMe	60
1	C=O	NH	460

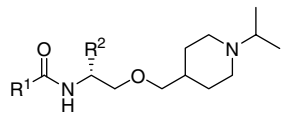
[a] K_{ass} is the apparent association constant reported in units of 10^6 L/mol and is approximately equal to $1/K_i$. K_{ass} values were obtained by the method of Smith et al.[9] and are the result of a single experiment (performed in triplicate at each of four to eight inhibitor concentrations).[10]

[b] Compound **11** was tested as a racemate.[11]



Scheme 2. Preparation of carbon-linked analog of **2**. Reagents and condition: (a) $HONH_2 \cdot HCl$, NaOAc, EtOH, 80 °C, 89%; (b) Pd/C, H_2 (60 psi), 87%; (c) Boc_2O, Et_3N, THF, 82%; (d) PtO_2, AcOH, H_2 (60 psi);(e) acetone, $NaCNBH_3$, AcOH, MeOH; (f) 4 N HCl, dioxane; (g) **6**, EDCl, HOAt, DMF, 20% (four steps).

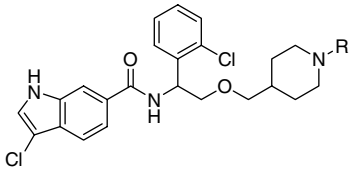
Table 2. Effect of substitution of R1 and R2 on binding to factor Xa



Compound	R^1	R^2	K_{ass} (10^6 L/mol)
2	3-Cl-6-indole	Ph	1700
14	3-Me-6-indole	Ph	160
15	6-Indole	Ph	35
16	4-MeO-Ph	Ph	3
17	3-Cl-6-indole	2-Cl-Ph	850[a]
18	3-Cl-6-indole	2-F-Ph	650[a]
19	3-Cl-6-indole	*i*-Pr	14
20	3-Cl-6-indole	*n*-Bu	41

[a] Compound was tested as a racemate.[11]

Table 3. Effect of piperidine substitution on binding to factor Xa



Compound	R	K_{ass} (10^6 L/mol)
17	*i*-Pr	850[a]
21	Et	150[a]
22	Me	65[a]
23	4-Pyridyl	1300[a]

[a] Compound was tested as a racemate.[11]

cyclic alkyl groups maintain useful affinity for the enzyme, isopropyl provides the greatest amount of binding affinity. It is interesting that the S4 pocket can accommodate a 4-pyridyl substituent and maintain high affinity. Modeling suggests that the pyridine ring may be able to interact with or displace a water molecule that is located at the bottom of the S4 pocket (Fig. 3).

The potential of phenylglycinol-derived inhibitors as therapeutic agents is further demonstrated by the in vitro anticoagulant profile of **2** (Table 4). Anticoagulant activity as measured by prolongation of prothrombin time shows that both ether and amide linkages provide inhibitors with similar activity when measured in human plasma. Interestingly, **2** provides improved anticoagulant activity in rat and dog plasma relative to its amide comparator **1**. The reason for the marked difference in preclinical species anticoagulant profile is unclear, however, it has been reported that the factor Xa sequence differs between species in a loop that resides near the S4 pocket.[12]

Table 4. Effect of linker on species in vitro anticoagulant profile[13]

Compound	Human 1.5XPT (μM)	Rat 1.5XPT (μM)	Dog 1.5XPT (μM)	Monkey 1.5XPT (μM)
2	0.4	0.6	0.8	0.4
1	0.5	2.6	3.2	0.6

In conclusion, replacement of the secondary amide linkage in specific D-phenylglycinamide derived factor Xa inhibitors with an alkyl ether linkage provides inhibitors with improved measured binding affinity for factor Xa. X-ray co-crystallography confirms the binding hypothesis around this series and can serve to refine future computational docking models for this series.

Acknowledgments

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6. The coordinate set for the protein structure used for the molecular modeling was the human factor Xa X-ray structure of Brandstetter et al.[14] (RCSB PDB file 1fax.pdb). The initial structure of inhibitor **1** was a model built starting from the related phenylglycine ligand contained in the trypsin X-ray structure 1eb2.pdb.[15] Using residue identity, the trypsin structure complex was superimposed onto the factor Xa protein structure, and then the ligand coordinates of the former were extracted and modified. The initial structure of **1** was then inserted into the factor Xa structure. Molecular modeling then proceeded in a similar fashion as detailed previously in Ref. 9a. All molecular modeling was performed with QUANTA and CHARMm, version 98 (Accelrys Inc., San Diego, CA 92121).

7. *For detailed experimental procedures*: Liebeschuetz, J. W.; Sheehan, S. M.; Watson, B. M. PCT Int. Appl. WO2004060872.
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