3-Substituted Imidazo[1,2-*d*][1,2,4]-thiadiazoles: A Novel Class of Factor XIIIa Inhibitors

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Abstract: A new class of selective FXIIIa inhibitors with a bicyclic [1,2,4]-thiadiazole pharmacophore is described. At 160 μM, compound **8** caused 50% reduction in fibrin γ-chain cross-linking and suppressed the polymerization of α chains in platelet-depleted human plasma clots. Fibrinolysis rates in response to tissue plasminogen activator were directly proportional to the concentration of **8** in plasma at the time of clotting.

Factor XIII (FXIII) is involved in the final step of the blood coagulation cascade, and its main function is to cross-link adjacent fibrin fibers into fibrin clot in response to an external or an internal injury to the blood vessels.[1−3] FXIII exists as a heterotetramer of two A and two B subunits in the circulating plasma and as a dimer of two A subunits in platelets, placenta, and other tissues. The X-ray crystal structures of several forms of FXIII A subunit revealed a conserved Cys314-His373-Asp396 triad in the active site.[4,5] The active site cysteine is not accessible for catalysis in the zymogen form.[5] In the presence of Ca^{2+}, thrombin mediates the conversion of FXIII to the active form FXIIIa. The large change in protein conformation that occurs upon enzyme activation allows access to the active site and hence the active site cysteine. Both the platelet and the plasma enzymes are identical upon activation.[2−6]

FXIIIa catalyzes the formation of amide bonds between γ-carboxamide of glutamine and N^{ϵ}-lysine residues of proteins and/or peptides to form the N^{ϵ}-(γ-glutamyl)lysine bond with release of ammonia.[1−3] Similarly, FXIIIa cross-links the γ chains of fibrin to form γ dimers and causes the polymerization of the α chains into high molecular weight structures. In addition, FXIIIa catalyzes the cross-linking of α_2-antiplasmin, a potent inhibitor of fibrinolysis, to the α chains of fibrin. The γ-chain dimerization and the cross-linking of α_2-antiplasmin are relatively fast processes and are completed within a few minutes after clot formation, while the polymerization of the α chains is much slower and takes several hours. As a result of FXIIIa action, the mechanical strength of the clot is improved, and its stability against fibrinolytic enzymes is enhanced. FXIIIa is also capable of cross-linking fibrin to extracellular matrix proteins such as fibronectin, vitronectin, and collagen, thereby anchoring the clot to the blood vessel wall.[2,3]

Chart 1. Examples of Non-Peptide Inhibitors of Factor XIIIa via Modification of Active Site Cysteine Thiol[13−15]

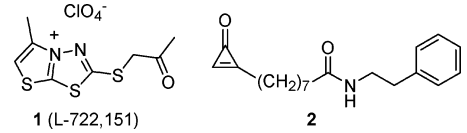
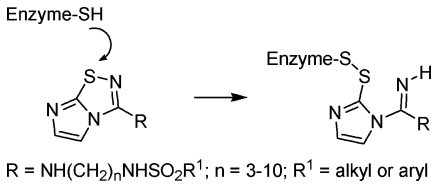


Chart 2. Inhibition of Thiol-Dependent Enzymes by 3-Substituted Imidazo[1,2-*d*][1,2,4]-thiadiazoles



Factor XIIIa inhibitors do not prevent fibrin clot formation, but they impede the formation of highly cross-linked, polymeric "hard" clots. Therefore, specific FXIIIa inhibitors can potentially provide a safer alternative to anticoagulants for the therapeutic treatment of thrombosis,[7] atherosclerosis,[8] and coronary heart diseases.[9] Several FXIIIa inhibitors (e.g., the polypeptide tridegin,[10] the low molecular weight non-peptide ZG-1400,[11] cerulenin,[12] and alutacenoic acids A and B[13,14]) have been discovered through screening of natural products. Surprisingly, very few specific non-peptide inhibitors of FXIIIa have been reported to date.[13−17] Interestingly, **1**[15,16] and **2**[13,14] (Chart 1) inactivate FXIIIa through modification of the active site thiol.

We[18] and others[19] have recently disclosed the monocyclic [1,2,4]-thiadiazoles as cysteine protease inhibitors. It has been postulated that the active site Cys314 of FXIIIa could be accessed from a long groove that runs along the subunit−subunit interface or by bending along the β-sandwich domain.[5] A binding form model of FXIIIa was also built using data from the X-ray crystal structure of the zymogen.[14] Furthermore, monotosylcadaverine (Tos-NH$(CH_2)_5NH_2$) and its derivatives are known potent pseudo substrate inhibitors of transglutaminases including FXIIIa.[20,21] We reasoned that compounds (Chart 2) that incorporated the [1,2,4]-thiadiazole pharmacophore[18,22] substituted at the 3 position with an amino anchor followed by a hydrophobic $(CH_2)_n$ (n = 3−10) spacer and terminated with an amino sulfone moiety could be promising FXIIIa inhibitors.

Herein, we describe specific inhibitors of FXIIIa based on a novel class of thiol-trapping pharmacophore, the 3-substituted imidazo[1,2-*d*]-1,2,4-thiadiazole, with a $(CH_2)_6$ spacer and report our initial findings on their inhibitory activities.

Compounds **4**−**10** were prepared uneventfully as shown in Scheme 1. These new compounds did not react with the zymogen FXIII but inhibited the activated FXIIIa enzyme. These bicyclic [1,2,4]-thiadiazoles **5**−**10** inhibited pure FXIIIa in a time-dependent, irreversible manner, and the loss of enzymatic activity followed

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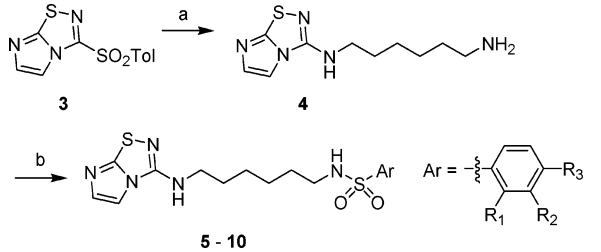
Scheme 1. Synthesis of 3-Substituted Bicyclic [1,2,4]-Thiadiazole Sulfonamide Derivatives[a]



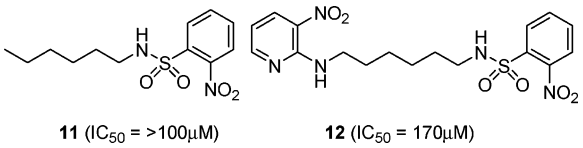
[a] Reagents and conditions: (a) 5 equiv of 1,6-hexanediamine, 2 equiv of Et_3N, DMF; (b) 2 equiv of $ArSO_2Cl$, pyridine or 1.1 equiv of $ArSO_2Cl$, 2 equiv of solid K_2CO_3, acetone.

Table 1. In Vitro Activity Data of the New Bicyclic [1,2,4]-Thiadiazole Derivatives toward Purified Factor XIIIa

compd	R_1	R_2	R_3	IC_{50} (μM)[a]
4				11.0
5	H	H	H	2.3
6	CH_3	H	H	0.7
7	H	H	CH_3	2.2
8	NO_2	H	H	0.13
9	H	NO_2	H	1.9
10	H	H	NO_2	0.91

[a] IC_{50} values were generated by linear regression of percent inhibition vs log inhibitor concentration for at least three different concentrations. Standard errors of the slope and the intercept were below 6%. Enzyme activation procedure and the assay conditions were as described earlier (see ref 15).

Chart 3. Examples of 2-Nitrobenzenesulfonamide Derivatives without the Thiadiazole Moiety and Their Associated Activity Data toward Factor XIIIa



pseudo-first-order kinetics. The inactivated enzyme did not regain its activity upon exhaustive dialysis. However, the enzymatic activity could be restored by incubation with dithiothreitol (DTT), a disulfide bond reducing agent. These observations are consistent with the proposed mechanism (Chart 2) of active-site directed inactivation of the enzyme brought about by the formation of a disulfide bond between the active site cysteine and the sulfur atom of the thiadiazole.[18]

The in vitro inhibitory results of **4**−**10** toward FXIIIa are shown in Table 1. Compound **4** inhibited FXIIIa with an IC_{50} of 11.0 μM.[15,23] The incorporation of a phenylsulfone moiety resulted in inhibitor **5**, and interestingly its potency toward FXIIIa has now increased by almost 5-fold compared to **4**. Moreover, the substitution pattern on the aryl ring attached to the sulfone moiety strongly influenced the inhibitory activities of these compounds (**6**−**10**) because inactivators bearing ortho substituents were the most effective against FXIIIa. In this series of compounds, inhibitor **8** with an IC_{50} of 130 nM was identified as the lead. Interestingly, **11** and **12** (Chart 3), which do not possess the bicyclic [1,2,4]-thiadiazole moiety, did not inhibit FXIIIa at up to 100 μM.

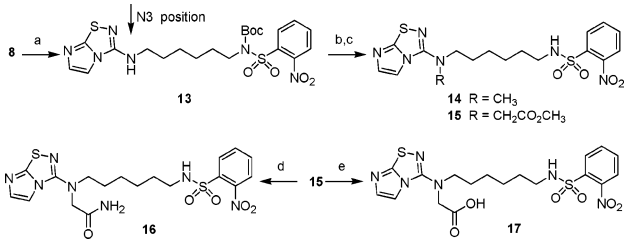
This class of small nonpeptidic compounds displayed selectivity for FXIIIa against the closely related transglutaminase. For example, **8** was slightly less potent

Table 2. Inhibitory Activities of Bicyclic [1,2,4]-Thiadiazole Derivatives toward FXIIIa and Guinea Pig Liver Transglutaminase and Their Aqueous Solubilities at pH 7.4

	FXIIIa		Tgase	ratio[d]	
compd	IC_{50} (μM)[a]	k_i/K_i (M^{-1} s^{-1})[b,c]	IC_{50} (μM)[a]	Tgase/ FXIIIa	solubility[e] (mg/mL)
1[f]	0.10	23000	0.25	2.5	NA[g]
8	0.13	17000	8.1	62	0.013
14	0.11	21000	6.2	56	0.004
16	0.42	6700	32	76	0.081
17	0.62	4500	17	27	0.380

[a] IC_{50} values were generated by linear regression of percent inhibition vs log inhibitor concentration for at least three different concentrations. Standard errors of the slope and the intercept were below 6%. [b] The second-order rate constants for inactivation were determined using the same assay procedure but varying the incubation time and inhibitor concentration as described by Kitz and Wilson (see ref 24). Standard errors of the slope and the intercept of linear regression of apparent rate constants for inhibition at a given inhibitor concentration vs inhibitor concentration were below 5%. [c] See ref 25. [d] The number in the table represents the preference of the inhibitor for FXIIIa over guinea pig liver transglutaminase and was calculated as a ratio of the appropriate IC_{50} values. [e] Solubilities of the inhibitors were measured at pH 7.4. [f] Compound **1** was prepared using a procedure described in ref 26. [g] Not available.

Scheme 2. Synthesis of 2-Nitrobenzenesulfonamide 3-Substituted Bicyclic [1,2,4]-Thiadiazole Derivatives[a]



[a] Reagents and conditions: (a) 1.5 equiv of Boc_2O, 0.5 equiv of DMAP, ACN; (b) for **14**, excess CH_3I, solid K_2CO_3, powder KOH, cat. n-$Bu_4N^+I^-$, toluene; for **15**, excess $BrCH_2CO_2CH_3$, solid K_2CO_3, powder KOH, cat. n-$Bu_4N^+I^-$, toluene; (c) HCl(g), MeOH, then neutralize; (d) concentrated NH_3, MeOH; (e) 3 N NaOH, MeOH, then HCl.

than Merck's L-722,151[15] (**1**, Table 2) but showed considerably higher selectivity against guinea pig liver transglutaminase[23] (62-fold) and cysteine-dependent proteases, caspase-1 (65-fold) and caspase-3 (35-fold) (results not shown). Furthermore, **8** inactivated pure FXIIIa with a second-order rate constant (k_i/K_i) of 17 000 M^{-1} s^{-1}, which is 2500 times greater than the second-order rate constant for its reaction with glutathione (6.8 M^{-1} s^{-1}).[24,25]

The aqueous solubility of inactivator **8** at pH 7.4 (0.013 mg/mL) was disappointing. Through a synthetic effort, **14**−**17** were prepared by regioselective alkylation of the amino group at the N3 position of the thiadiazole moiety via a protection−alkylation−deprotection protocol (Scheme 2). The structures of the new compounds **14**−**17** were characterized by 2D NMR, including the HSQC and HMBC experiments.

Introduction of a methyl substituent at the N3 position of the thiadiazole moiety resulted in **14**, which displayed inhibitory activities comparable to that of inhibitor **1** toward FXIIIa but exhibited a 56-fold preference over transglutaminase (Table 2). However, a 3-fold loss in solubility relative to inhibitor **8** was observed for **14** on substituting H for Me. Functionalization of the methyl substituent (CH_2−H) in **14** with a more polar

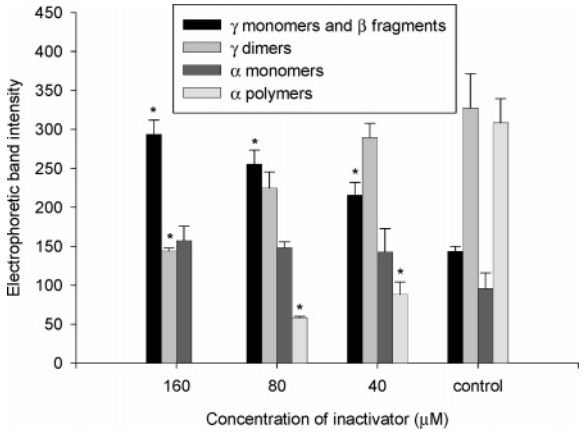


Figure 1. Composition of platelet-depleted human plasma clots made in the presence of varying concentrations of the bicyclic [1,2,4]-thiadiazole inhibitor (**8**). Each bar represents an average of three experiments. Mean values that are significantly different from the mean of an appropriate control (Student's t test, $p < 0.003$) are indicated by an asterisk (∗).

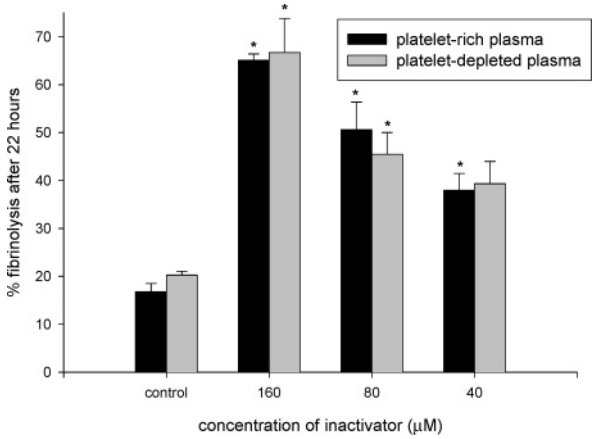


Figure 2. Acceleration of fibrinolysis of platelet-depleted and platelet-rich human plasma clots made in the presence of varying concentrations of inhibitor (**8**). Citrated human plasma (0.3 mL) spiked with ^{125}I-fibrinogen (200 μCi/mg) was clotted in the presence or absence of inhibitor by the addition of $CaCl_2$ (50 mM) and thrombin (5 units/mL). After a 30-min incubation at 37 °C, clots were harvested by winding on a bamboo stick and washed three times in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 1 mM EDTA. Clots were placed in 2 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, and 300 IU/mL human t-PA and were lysed at 37 °C for 22 h. The degree of fibrinolysis was measured as a percent of ^{125}I-fibrinogen solubilized from clots. Each bar represents an average of three experiments except for 40 μM **8** in platelet-depleted plasma where $n = 2$. Mean values that are significantly different from the mean of an appropriate control (Student's t test, $p < 0.0007$) are indicated by an asterisk (∗).

group such as CH_2-CONH_2 (**16**) or CH_2-COOH (**17**) caused a 3- to 4-fold decrease in potency for these inhibitors toward FXIIIa (Table 2). However, the acid derivative **17** showed an almost 30-fold increase in aqueous solubility at pH 7.4 (0.38 mg/mL) relative to inactivator **8** (0.013 mg/mL). The second-order rate constant ($k_i/K_i = 4500\ M^{-1}\ s^{-1}$) measured for **17** was very similar to that reported for inhibitor **2** ($k_i/K_i = 5083\ M^{-1}\ s^{-1}$) shown in Chart 1.[13]

We next turned our attention to the effect of our compounds on the composition of platelet-depleted plasma clots (Figure 1). Plasma spiked with ^{125}I-fibrinogen was clotted at 37 °C for 2 h in the presence and the absence of varying concentrations of inhibitor **8**. The clots were washed, dissolved in 8 M urea with 10% SDS and 10% mercaptoethanol, and analyzed using SDS−PAGE.[15] Electrophoretic bands were quantitated by autoradiography. Increasing inhibitor concentration resulted in a decrease in the formation of γ dimers and higher molecular weight fibrins. The formation of α polymers was completely suppressed at 160 μM concentration of **8**. At that same inhibitor concentration, the amount of γ dimers has been halved relative to the no-inhibitor control. Clots, formed from ^{125}I-fibrinogen spiked platelet-depleted and platelet-rich human plasma in the presence of varying concentrations (40, 80, and 160 μM) of inhibitor **8**, were subjected to in vitro fibrinolysis by human tissue plasminogen activator (t-PA).[15] The amount of lysed fibrin was measured after 22 h at 37 °C. Plasma clots were carefully washed to remove the inhibitor and were placed in a fresh solution containing t-PA. This design of fibrinolysis studies precludes the possibility of inhibitors directly affecting the interaction between t-PA and plasminogen activator inhibitor-1 (PAI-1) or plasmin and α_2-antiplasmin because it is not present in solution at the time of fibrinolysis.

Figure 2 illustrates the acceleration of fibrinolysis with increasing inhibitor concentration relative to no-inhibitor control. These results and the analysis of composition of the clots made in the presence of inhibitor **8** strongly suggest that the acceleration of in vitro fibrinolysis is due to the inhibition of FXIIIa-catalyzed processes.

Detailed results on the biological studies of this new class of FXIIIa inhibitors will be reported in due course.

Supporting Information Available: Synthesis and spectral data of the new inhibitors, including 2D-COSY, 2D-HSQC, and 2D-HMBC spectra of inhibitor **17**. This material is available free of charge via the Internet at http://pubs.acs.org.

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