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CHARACTERIZATION OF LY806303 AS A POTENT AND SELECTIVE INHIBITOR OF THROMBIN

Daniel J. Sall,* Dennis R. Berry, William J. Coffman, Trelia J. Craft, Michael L. Denney, Donetta S. Gifford-Moore, Marcia L. Kellam, and Gerald F. Smith

Lilly Research Laboratory, Eli Lilly and Company
Indianapolis, IN 46285

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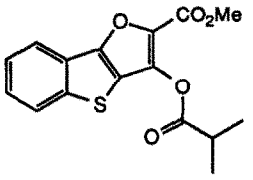
Abstract: Methyl 3-(2-methyl-1-oxopropoxy)[1]benzothieno[3,2-b]furan-2-carboxylate (LY806303; **1**) has been characterized as a novel, potent and selective inhibitor of thrombin.

Thrombin, a trypsin-like serine protease, plays a pivotal role in the development of thrombosis through the stimulation of fibrin formation and platelet aggregation.[1] Current clinical regulation of thrombosis involves the administration of heparin[2,3] or warfarin.[4] However, each of these agents suffers from well-documented liabilities[5,6] which has stimulated an intense effort to develop safe and effective direct-acting inhibitors of thrombin.

During fibrin formation, thrombin cleaves an Arg-Gly bond in both the A and B chains of fibrinogen.[1] Classical routes to the design and synthesis of active-site-directed inhibitors of thrombin[7] have employed groups containing a highly basic guanidine or amidine as an arginine surrogate resulting in a number of potent synthetic agents.[8-14] However, a number of coagulation factors and fibrinolytic enzymes are also arginine- or lysine-directed endopeptidases which has made the development of thrombin specific agents difficult. Irreversible inhibitors which lack a positively charged recognition element, yet target the active site serine, have also been studied.[15] Few, however, display thrombin specificity over the myriad of other physiologically relevant serine proteases. This communication will detail the preliminary characterization of methyl 3-(2-methyl-1-oxopropoxy)[1]benzothieno[3,2-b]furan-2-carboxylate (LY806303; **1**) as a neutral yet extremely potent and selective small molecule inhibitor of thrombin.



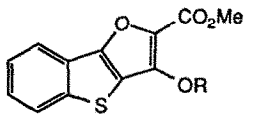
LY806303; 1

Methyl ester **1** and its derivatives (**Table I**) were prepared according to literature procedures. Commercially available methyl o-nitrobenzoate was converted to methyl 3-hydroxy-[1]-benzothieno[3.2-b]furan-2-carboxylate (LY110645; **2**) according to the method of Beck.[16,17] Subsequent derivatization of the C-3

hydroxyl using isobutyryl chloride, dimethyl sulfate or dimethylcarbamyl chloride afforded LY806303 (**1**),[18] LY110646 (**3**),[17] and LY282388 (**4**)[19] respectively.

Analogues **1-4** were initially evaluated *in vitro* for their ability to inhibit the thrombin catalyzed hydrolysis of a the synthetic substrate Bz-Phe-Val-Arg-p-nitroanilide (column 1; **Table I**).[20] At submicromolar concentrations, compound **1** inhibited thrombin with the same potency demonstrated by the peptidyl inhibitor D-MePhg-Pro-Arg-H.[8] Altering the electronic nature of the C-3 isobutoxy substitutuent as in analogue **4** or

Table I: Thrombin Inhibition by 3-Substituted Methyl [1]-Benzothieno[3.2-b]furan-2-carboxylates (**1-4**).[20]



1-4

		2x TT (ug/mL)[b]		
Compound	IC_{50} (μM)[a]	Buffer	Buffer+BSA	Ci-Trol
1: R = $COCH(Me)_2$	0.03	0.09	1.7	> 44.6
2: R = H	> 10	nt[c]	nt	nt
3: R = Me	> 10	nt	nt	nt
4: R = $CONMe_2$	> 10	nt	nt	nt
D-MePhg-Pro-Arg-H	0.02	0.10	nt	0.08

[a]The concentration of inhibitor required to cause 50% inhibition of 6 nM enzyme (n=3).
[b]The concentration of inhibitor necessary to cause a doubling in the time to clot formation (n=3).
[c] nt: not tested.

replacing it with a simple hydrogen or methyl (**2** and **3** respectively) results in a >30-fold loss in activity suggesting, a critical need for an electrophilic substituent attached to the C-3 hydroxyl.

Under more physiologically relevant conditions, inhibitor **1** was evaluated *in vitro* as an inhibitor of the thrombin catalyzed conversion of human fibrinogen to fibrin (columns 3-5; **Table I**).[21] In buffer alone, compound **1** blocked fibrin formation at concentrations of 0.09 μg/mL, compared to 0.1 μg/mL for D-MePhg-Pro-Arg-H. However, when physiologically relevant levels of BSA were added to the assay mixture, 20 times more compound was required to produce the same degree of inhibition. Finally, no activity was detected in reconstituted human plasma (Ci-Trol^R) even at concentrations exceeding 40 μg/mL. The 20-fold loss of activity in the presence of BSA suggests that the inhibitor binds tightly to plasma albumen which may explain, in part, why this analogue is inactive in reconstituted plasma. However, the >25-fold loss in activity in going from buffer+BSA to plasma, suggests that compound **1** also interacts with other plasma constituents that are yet to be defined.

Structural considerations show that inhibitor **1** possesses two labile acyl groups which could chemically react with thrombin. Irreversible inhibition through nucleophilic attack at either the C-3 isobutoxy substituent or the C-2 methyl ester could generate a catalytically-inactive protein. In order to test the reactivity of compound **1** towards thrombin, the inhibitor was preincubated in Tris buffer in the presence and absence of thrombin (**Figure**

1).[22] The degree of inhibition, measured as a percent of control, was plotted as a function of incubation time. Upon preincubation with thrombin, a time-dependant decrease in inhibitory activity was observed, suggestive of enzyme-catalyzed inhibitor degradation. Acylation of thrombin to form an inactive enzyme/acyl complex which can then be subsequently hydrolyzed back to native enzyme, is consistent with the time dependant decrease in inhibition observed upon preincubation of the inhibitor with thrombin. Current studies are aimed at elucidating the site on **1** at which thrombin reacts.

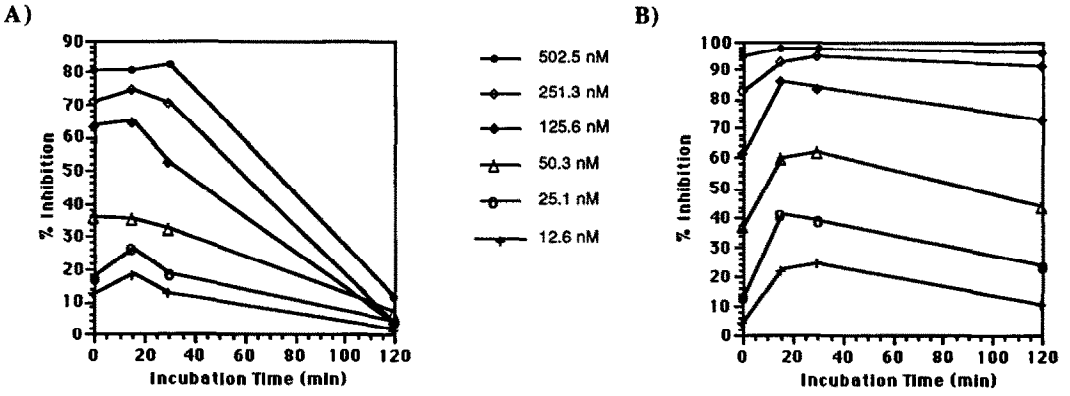


Figure 1: Degradation of **1** in the A) presence and B) absence of bovine thrombin.[22]

Acylating agents such as aspirin, can be clinically effective drugs provided they possess the necessary reactivity and specificity. While a number of inhibitors which acylate thrombin have been reported,[15] few possess the necessary thrombin specificity to be valuable. The protease inhibitor specificity of compound **1** was studied with respect to other serine proteases: including those relevant to blood coagulation such as plasmin, Xa, and tissue plasminogen activator (rt-PA); and the enzymes trypsin and kallikrein (**Table II**).[23]

Table II: Inhibition of Serine Proteases by **1**[23]

	IC_{50} (μM)[a]					
Compound	Thrombin	Trypsin	Plasmin	Factor Xa	Kallikrein	nt-PA
1	0.03	29	240	0.10	7.2	160
D-MePhg-Pro-Arg-H	0.02	0.02	0.50	2.2	12	43

[a]The average concentration of inhibitor required to cause 50% enzyme inhibition (n=3).

Inhibitor **1** selectively inhibited thrombin over these serine proteases by factors ranging from 50 to 10,000 and was more selective in this respect than D-MePhg-Pro-Arg-H.[8] Most significantly, compound **1** only weakly inhibited the thrombolytic enzymes plasmin and nt-PA, suggesting that the inhibitor could block clot formation, without interfering with the endogenous clot lysis system. In addition, the low activity expressed towards nt-PA suggests that an agent such as analogue **1** could be used as an adjunct to thrombolytic therapy without interfering with the exogenous serine protease.

In conclusion, compound **1** has been prepared and characterized as a potent and highly selective inhibitor of the serine protease thrombin. *In vitro*, it blocks the ability of thrombin to catalyze the hydrolysis of fibrinogen resulting in a prolongation of the time to clot formation. Structural considerations in conjunction with preliminary preincubation studies suggest that thrombin inhibition occurs via enzyme acylation. The inhibitor does not, however, appear to serve as a general acylating agent since it selectively inhibits thrombin with little affinity for the serine proteases considered in this study. While analogue **1** displays excellent affinity and selectivity for thrombin, it appears to interact with plasma constituents which limits the anticoagulant activity in physiological media. The challenge is to modify the structural features of the inhibitor to overcome the protein interactions while retaining high thrombin affinity and specificity. The results of this endeavor, as well as further studies on the mechanism of thrombin inhibition, will be addressed in future publications.

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18) For compound **1**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.42 (d, 6H), 2.97 (s, 1H), 3.98 (s, 3H), 7.40-7.62 (m, 2H), 7.78-7.86 (m, 1H), 8.02-8.05 (m, 1H); FDMS 318 (M+); Anal. Calcd for $C_{16}H_{14}O_5S$: C, 60.37; H, 4.43. Found: C, 60.47; H, 4.45.
19) For compound **4**: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.97 (s, 3H), 3.13 (s, 3H), 3.87 (s, 3H), 7.45-7.56 (m, 2H), 8.00-8.09 (m, 2H); FDMS 319 (M+); Anal. Calcd for $C_{15}H_{13}NO_5S$: C, 56.42; H, 4.10; N, 4.39. Found: C, 56.20; H, 3.97; N, 4.39.
20) The degree of inhibition was determined by monitoring the increase in absorbance at 405 nm caused by the release of p-nitroanilide when bovine thrombin (19.3 nM; Parke-Davis) catalyzed the hydrolysis of Bz-Phe-Val-Arg-pNA (0.2 mM).
21) Inhibitory activity was determined in 3 different media including TRIS buffer, TRIS buffer containing 30 mg/mL BSA, and reconstituted plasma (Ci-Trol^R; Dade Laboratories, Miami). In the buffer and buffer/BSA assays, a solution of the inhibitor and 10 μM fibrinogen is treated with 19.3 nM thrombin and the time to clot formation is measured as a function of opacity using a Coa SCREENER (American Labor; Durham, NC). In reconstituted plasma, a mixture of inhibitor in Ci-Trol^R is treated with 19.3 nM bovine thrombin and the time to clot formation recorded.
22) Thrombin catalyzed inhibitor degradation was determined by incubating 6 concentrations of **1** at 25 °C for 0, 15, 30, and 120 min in the presence of 5.8 nM bovine thrombin or TRIS buffer. After incubation, TRIS buffer and 5.8 nM thrombin was added to the respective assay mixtures and the residual thrombin inhibitory acitivity of **1** measured according to reference 9.
23) Enzyme specificity data was generated according to the proceedure in ref. 9 using the following enzymes/substrate systems: Bovine Trypsin (1.4 nM) / Bz-Phe-Val-Arg-p-nitroanilide (0.18 mM); Human Plasmin (3.4 nM)/ H-D-Val-Leu-Lys-p-nitroanilide (0.5 mM); Bovine Factor Xa (1.3 nM) / Bz-Ile-Glu-Gly-Arg-p-nitroanilide (0.2 mM); Human plasma kallikrein (0.27 nM)/ H-D-Pro-Phe-Arg-p-nitroanilide (0.8 mM); Human nt-PA (1.2 nM) / H-D-Ile-Pro-Arg-p-nitroanilide (0.82 mM).