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Thrombin Active Site Inhibitors: Chemical Synthesis, In Vitro and In Vivo Pharmacological Profile of a Novel and Selective Agent BMS-189090 and Analogues

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Abstract—A series of structurally novel small molecule inhibitors of human α-thrombin was prepared to elucidate their structure–activity relationships (SAR), selectivity and activity in vivo. BMS-189090 (**5**) is identified as a potent, selective, and reversible inhibitor of human α-thrombin that is efficacious in vivo in a mice lethality model, and in inhibiting both arterial and venous thrombosis in a rat model.

Thrombin is the serine protease that occupies the central position in the blood coagulation cascade.[1] It proteolytically cleaves A-α and B-β chains of fibrinogen with release of fibrin monomer which are polymerized and covalently linked by thrombin activated factor XIIIa. In addition thrombin amplifies the coagulation cascade by activating factors V and VIII, and activates platelets through its surface receptors leading to platelet shape change and aggregation.[2] Accordingly, direct inhibition of thrombin has attracted considerable attention as a therapeutic target for the management of thrombotic diseases including arterial and venous thrombosis, transient ischemic attack, stroke and coronary artery bypass surgery.[3]

Over the past decades development of direct acting thrombin inhibitors[4] has been an area of intense research. Our drug discovery effort in this arena has focused on discovering structurally novel, reversible and direct acting inhibitors that have no electrophile. By combining crystallographic data from the structure of Argatroban with trypsin,[5] and the initial SAR of a novel series of tripeptide inhibitors,[6] we designed a new template for building thrombin active site inhibitors (Fig. 1). This template is widely amenable to synthetic modification, and has provided a motif from which new thrombin inhibitors have been developed. We have been able to modify these compounds to obtain highly potent and selective reversible inhibitors of thrombin active site that demonstrate good antithrombotic activity in vivo.

The synthesis of this class of compounds follows a general synthetic route that is illustrated in Scheme 1 with the preparation of **5** (BMS-189090).

Treatment of tosylate **1**[7] with 1.5 equiv of sodium azide followed by removal of BOC-protecting group and subsequent coupling of the crude amine with N^{α}-BOC-L-benzyloxyserine afforded azide **2** in 92% yield.

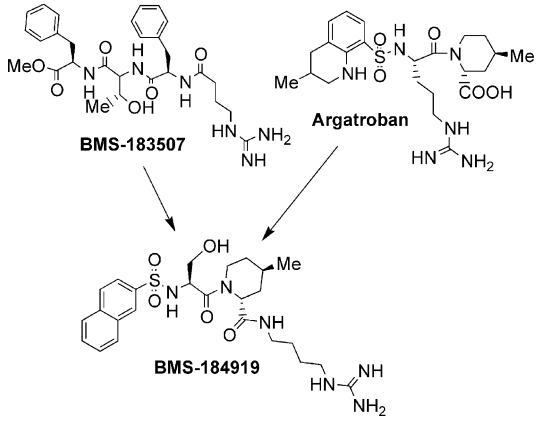

Figure 1. Schematic representation of novel inhibitor design from Argatroban and BMS-183507.

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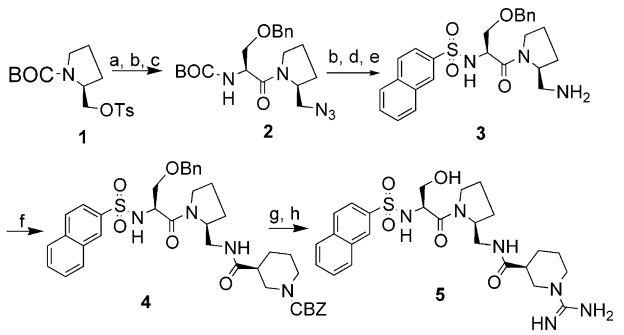


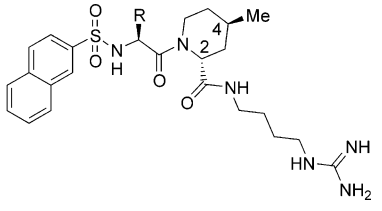
Scheme 1. Synthesis of **5**; (a) NaN_3, DMSO, 70 °C; (b) TFA, CH_2Cl_2, 0 °C; (c) EDAC, HOBt, BOC-L-Ser(OBn)-OH, DMF, NMM; (d) CH_2Cl_2, Et_3N, naphthalene-2-sulfonyl chloride; (e) 10% Pd–C, H_2, EtOH; (f) EDAC, HOBt, (*S*)-*N*-CBZ-nipecotic acid, NMM, DMF; (g) 10% Pd–C, H_2 (55 psi), EtOH, AcCl; (h) DMF, *i*-Pr_2NEt, pyrazole-1-carboxamidine.

BOC-deprotection followed by reaction of the crude amine with naphthalene-2-sulfonyl chloride and subsequent hydrogenation of the chromatographically purified azide formed amine **3** in 66% yield. Coupling of the crude amine **3** with (*S*)-N^{α}-CBZ-nipecotic acid[8] formed amide **4** in 77% yield. Removal of CBZ and benzyloxy protecting groups under hydrogenolysis conditions, followed by reaction of the amine with pyrazole-1-carboxamidine[9] in DMF in presence of Hunig's base afforded **5** (BMS-189090) in 64% overall yield after purifications by reverse-phase preparative HPLC.

Compound **5** and its analogue were tested for their ability to inhibit thrombin hydrolysis[10] of the chromogenic substrate S-2238 (IC_{50}, Tables 1–5).

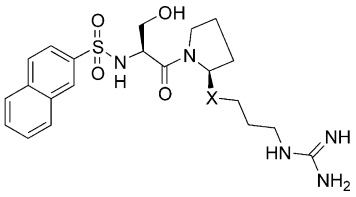
The prototypical analogue **6** (BMS-184919, Fig. 1) of the series has modest potency against thrombin in vitro. In contrast, the diastereomeric analogue **7** is roughly 3-fold more potent than **6**. The preferred stereochemistry of the azacycloalkyl ring at P_2 (2*S*,4*S*) is the mirror image of the preference anticipated from the solid state structure of Argatroban. Removal of the hydroxymethyl group at P_3 in both **6** and **7** resulted in a substantial drop in potency. More importantly substitution of the pipecolic acid moiety at P_2 of **7** with synthetically more accessible S-proline (**10**) resulted in only 5-fold drop in activity (Table 1). Accordingly further optimization studies were undertaken with **10**.

Table 1. SAR from the P_2 azacycloalkyl modification



Compd	R	Stereochemistry	Thrombin IC_{50} (μM)[10]
6	CH_2OH	2*R*,4*R*	1.7
7	CH_2OH	2*S*,4*S*	0.62
8	H	2*R*,4*R*	27
9	H	2*S*,4*S*	2.9
10	CH_2OH	S-Pro	2.8

Table 2. SAR from the P_1–P_2 linker modification



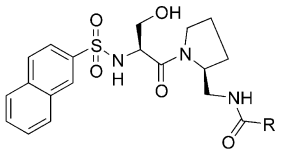
Compd	X	Thrombin IC_{50} (μM)[10]	Compd	X	Thrombin IC_{50} (μM)[10]
10	$CONHCH_2$	2.8	**14**	$CH_2NHCOCH_2$	0.08
11	CH_2SCH_2	1.4	**15**	$CH_2NHCO(CH_2)_2$	0.05
12	CH_2S	7.4	**16**	$CH_2NHCO(CH_2)_3$	14
13	CH_2NHCO	0.39			

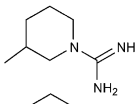
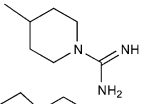
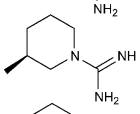
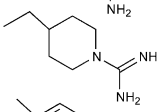
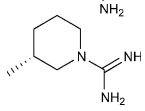
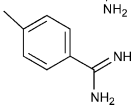
Table 2 outlines the SAR observed with the modification of the side chain connecting the pyrrolidine ring at P_2 and the guanidine moiety that binds to an aspartic acid residue (Asp) in the specificity pocket of thrombin. Replacement of the $-CONHCH_2-$ linker of **10** with thioether linkages (**11** and **12**) has a modest effect in thrombin inhibitory activity in vitro. In contrast, replacement by a reverse amide linker $-CH_2NHCO-$ (**13**) resulted in a 7-fold increase in activity. In this reverse amide series 4-5 methylene linker (**14** and **15**) appears to be optimal for thrombin inhibitory activity.

In order to further optimize the potency we investigated the effect of introducing a constrained hydrophobic ring binding to the P_1 specificity pocket of thrombin (Table 3). Introduction of a 3-substituted piperidine ring (**17**, mixture of diastereomers) resulted in a 12-fold increase in activity relative to **13**. The corresponding 4-substituted piperidine analogue **19** is 3-fold more potent than **7**. Synthesis of the enantiomerically pure diastereomers of **17** (**5** and **18**) demonstrates that the thrombin inhibitory activity resides primarily with the *S*-nipecotamide analogue **5** (BMS-189090). Extending the linker chain by a methylene unit in both **17** and **19** (data not shown) significantly attenuated the activity in vitro while the 4-benzamidine analogue **21** is found to be equipotent to **5** which is attributed to the increased hydrophobic interactions of the phenyl ring and strong hydrogen bond interaction of the amidine moiety with Asp189 residue in the P_1 specificity pocket of thrombin.

Further optimization on activity of **5** relied on modification of P_3 substituent, and is reported in Table 4. Substitution of the P_3 α-carbon with either hydrogen or a methyl group (**22** and **23**) significantly reduced the activity relative to the hydroxymethyl substituted analogue **5**. Neither the threonine or *allo*-threonine derived analogues (**24** and **25**, respectively) were as potent as **5**. In contrast the methylaspartate derived analogue **26** was found to be equipotent to **5**. Presumably the carbomethoxy group in **26** is engaged in a similar kind of hydrogen bond interaction as the hydroxymethyl function in **5** with backbone Gly219 of thrombin.[12]

Table 3. SAR from the P_1–P_2 linker modification



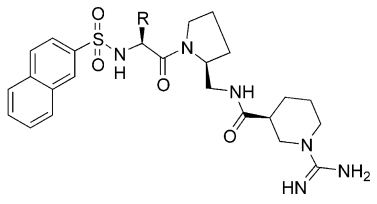
Compd	R	Thrombin IC_{50} (μM)[10]	Compd	R	Thrombin IC_{50} (μM)[10]
17		0.032	**19**		0.11
5		0.016	**20**		0.20
18		0.50	**21**		0.013

Selected inhibitors of this class were evaluated for their specificity over other serine proteases including trypsin, *t*-PA, plasmin and Factor Xa (Table 5). A clinically useful thrombin inhibitor should not modulate the fibrinolytic processes through inhibition of plasmin and *t*-PA.

As shown in Table 5, several analogues in this series (**5**, **13**, and **21**) exhibited excellent selectivity over other serine proteases. Of particular interest is **5** (BMS-189090), which is the most potent and most selective thrombin inhibitor in the series. The selectivity profile of BMS-189090 is comparable to that of Argatroban and significantly superior to that of Efegatran. The crystal structure of the ternary complexes of human α-thrombin with hirugen and BMS-189090 showed that this inhibitor binds in an antiparallel fashion to thrombin, like PPACK and Argatroban. These findings were reported in a previous communication.[12]

Because of its potency and selectivity, BMS-189090 was selected for further evaluations in vitro and in vivo. In a thrombin-induced lethality model in anesthetized mice[13] the in vivo potency of BMS-189090 ($ID_{50} = 0.11$ mpk, iv; 29 mpk po) was found to be similar to that of Efegatran ($ID_{50} = 0.21$ mpk, iv; 23 mpk po) and significantly superior to that of Argatroban ($ID_{50} = 1.6$ mpk, iv; >100 mpk po). BMS-189090 competitively and reversibly inhibited thrombin in vitro ($K_i = 3.44 \pm 0.05$ nM)[14] without showing time dependent kinetics like Efegatran which is characterized as a slow-binding tight inhibitor. In gel-filtered platelets BMS-189090 inhibited thrombin induced platelet aggregation with a pA_2 value of 8.61 ($n = 2$). A modified thrombin time (TT) was used to determine the direct inhibition of thrombin activity in a protein-rich plasma environment. In this assay, BMS-189090 doubled thrombin clotting time in vitro at 61 ± 2.3 nM ($n = 3$).

Table 4. SAR from the P_3 modification



Compd	R	Thrombin IC_{50} (μM)[10]	Compd	R	Thrombin IC_{50} (μM)[10]
22	H	0.31	**24**	CH(Me)OH (S)	0.65
23	CH_3	0.075	**25**	CH(Me)OH (R)	0.50
5	CH_2OH	0.018	**26**	CH_2COOMe	0.012

Table 5. Enzyme selectivity of selected thrombin inhibitors[11]

Compd	Thrombin IC_{50} (μM)[10]	Trypsin[a]	Plasmin[a]	tPA[a]	Factor Xa[a]
13	0.39	8	500	>800	300
5	0.018	8000	>18,000	>18,000	500
21	0.013	4	200	700	200
Argatroban	0.038	658	4900	6900	>1700
Efegatran	0.018	0.4	13,000	200	300

[a]Selectivity ratio: IC_{50}/IC_{50} (thrombin).

In pentobarbital-anesthetized rats BMS-189090 inhibited thrombosis in a dose-dependent manner (Fig. 2). Thrombosis was induced in either the carotid artery by topical $FeCl_2$ application or in the vena cava by a combination of blood stasis and endothelial disruption caused by hyptonic saline infusion.[15] Near complete inhibition of venous thrombosis (98%) was obtained at a dose of 0.5 mg/kg + 25 μg/kg/min iv. A lesser degree of

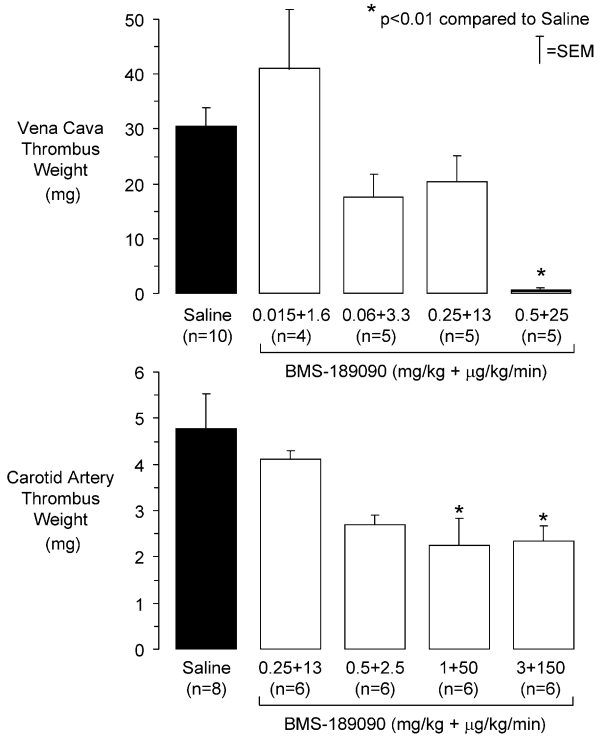


Figure 2. Effect of BMS-189090 on venous and arterial thrombosis in anesthetized rats.

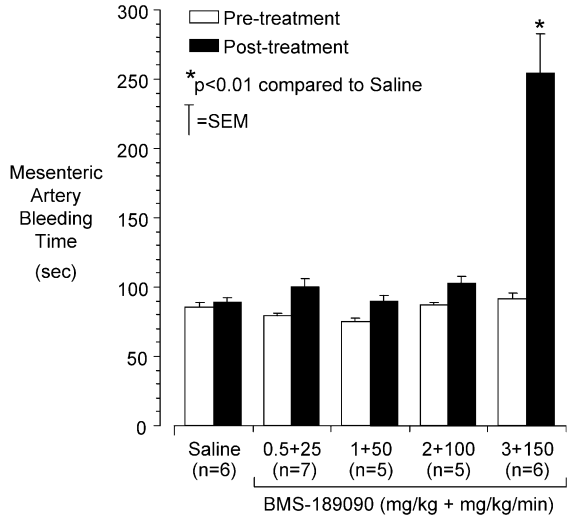


Figure 3. Effect of BMS-189090 on bleeding time in anesthetized rats.

inhibition of arterial thrombosis (53%) required a higher dose of 1 mg/kg + 50 μg/kg/min iv. The efficacy of BMS-189090 in arterial thrombosis was comparable to that obtained with Argatroban.[15] BMS-189090 also increased bleeding time by 180% in mesenteric arteries that were punctured with a hypodermic needle,[15] but the dose required to significantly increase bleeding time (3 mg/kg + 150 μg/kg/min iv) exceeded maximal doses for inhibition of venous and arterial thrombosis (Fig. 3).

In conclusion, we have described SAR of a structurally novel series of compounds leading to BMS-189090 as potent, highly selective, and reversible inhibitor of thrombin. The data provided show that BMS-189090 is efficacious in protecting mice from thrombin-induced lethality, in vivo and in inhibiting arterial and venous thrombosis in rats.

References and Notes

1. Colman, R. W.; Marder, V. J.; Salzman, E. W.; Hirsh, J. In *Hemostasis and Thrombosis*; Colman, R. W., Marder, V. J., Salzman, E. W., Hirsh, J., Eds.; Lippincott: Philadelphia, 1994; pp 3–18.
2. Coughlin, S. R. *Nature* **2000**, *407*, 258.
3. Vacca, J. P. *Curr. Opin. Chem. Biol.* **2000**, *4*, 394. Stein, B.; Fuster, V.; Halperin, J. L.; Chesebro, J. H. *Circulation* **1989**, *80*, 1501.
4. For recent reviews, see: Das, J.; Kimball, S. D. *Bioorg. Med. Chem.* **1995**, *3*, 999. Kimball, S. D. *Handb. Exp. Pharmacol.* **1999**, *132*, 367. Menear, K. *Curr. Med. Chem.* **1998**, *5*, 457. Coburn, C. A. *Exp. Opin. Ther. Pat.* **2001**, *11*, 721. Steinmetzer, T.; Hauptmann, J.; Sturzebecher, J. *Exp. Invest. Drugs* **2001**, *10*, 845.
5. Bode, W.; Huber, R.; Rydel, T. J.; Tulinsky, A. In *Thrombin Structure and Function*; Berliner, L. J., Ed.; Plenum: New York; p 3. Stubbs, M. T.; Bode, W. *Perspect. Drug Discov. Des.* **1994**, *1*, 431.
6. Tabernero, L.; Chang, C. Y.; Ohringer, S. L.; Lau, W. F.; Iwanowicz, E. J.; Han, W.-C.; Wang, T. C.; Seiler, S. M.; Roberts, D. G. M.; Sack, J. S. *J. Mol. Biol.* **1995**, *246*, 14.
7. Das, J.; Floyd, D. M.; Kimball, S. D.; Duff, K. J.; Lago, M. W.; Krapcho, J.; White, R. E.; Obermeier, M. T.; Moreland, S.; McMullen, D.; Normandin, D.; Hedberg, S. A.; Schaeffer, T. R. *J. Med. Chem.* **1992**, *35*, 2610.
8. (*S*)-N^{α}-CBZ-nipecotic acid was prepared from (−)-ethylnipecotate. D-Tartarate salt in two steps: (a) K_2CO_3, CBZ-Cl, Et_2O–H_2O, and (b) MeOH, 1 N aq NaOH. For preparation of (−)-ethylnipecotate. D-Tartarate see: Akkerman, A. M.; DeJongh, D. K.; Veldstra, H. *Receuil* **1951**, *70*, 899.
9. Bernatowicz, M. S.; Wu, Y.; Matsueda, G. *J. Org. Chem.* **1992**, *57*, 2497.
10. In vitro inhibition of thrombin catalytic activity using 10 μM substrate s-2238 (D-Phe-Pip-Arg-pNA) was measured at rt after 3 min incubation with inhibitor. For a description of the assay, see: Balasubramanian, N.; St. Laurent, D. R.; Federici, M. E.; Meanwell, N. A.; Wright, J. J.; Schumacher, W. A.; Seiler, S. M. *J. Med. Chem.* **1993**, *36*, 300.
11. Inhibition of bovine pancreatic trypsin was measured in an assay containing 2 mM $CaCl_2$, 50 mM Tris pH 8.0 and 30 μM carbobenzyloxy-Val-Gly-Arg-pNA as substrate. The inhibitor was combined with trypsin assay buffer and incubated for 3 min, after which the substrate was added and absorption was measured using a microplate reader (Molecular devices V_{max}) at 405 nM. Inhibition of human plasmin was measured in an assay containing 145 mM NaCl, 5 mM KCl, 1 mg/mL polyethylene glycol (PEG-8000), 30 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, pH 7.4 and 100 μM S-2251 (D-Val-Leu-Lys-pNA). The inhibitor was incubated with the enzyme for 3 min, after which the substrate was added and the rate of hydrolysis was measured as for thrombin and trypsin. Inhibition of tissue plasminogen activator (*t*-PA) was measured in an assay similar to that of human plasmin except using 100 μM methanesulfonyl-D-cyclohexyltyrosyl-Gly-Arg-pNA as substrate. Inhibition of Factor Xa was measured in an assay similar to that of human plasmin except using 100 μM S-2222 as substrate.
12. Malley, M. F.; Tabernero, L.; Chang, C. Y.; Ohringer, S.; Roberts, D. G. M.; Das, J.; Sack, J. S. *Protein Sci.* **1996**, *5*, 221.
13. The model involved iv challenge with human α-thrombin (10–20 units/mouse) in anesthetized mice treated with the inhibitor either 10 min (iv) or 60 min (po) prior to thrombin challenge. ID_{50} is the concentration of the inhibitor required for 50% mice survival.
14. K_i was determined by measuring enzyme activity at three different substrate (S) concentrations and six different inhibitor (I) concentrations, curve fitting the data to $V = V_{max}[S]/\{K_m(1 + [I]/K_i) + [S]\}$.
15. The experimental rats models were performed as described in: Schumacher, W. A.; Heran, C. L.; Steinbacher, T. E. *J. Cardiovasc. Pharmacol.* **1996**, *28*, 19.