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Low molecular weight thrombin inhibitors with excellent potency, metabolic stability, and oral bioavailability

Matthew M. Morrissette,[a,*] Kenneth J. Stauffer,[a] Peter D. Williams,[a] Terry A. Lyle,[a] Joseph P. Vacca,[a] Julie A. Krueger,[b] S. Dale Lewis,[b] Bobby J. Lucas,[b] Bradley K. Wong,[c] Rebecca B. White,[c] Cynthia Miller-Stein,[c] Elizabeth A. Lyle,[d] Audrey A. Wallace,[d] Yvonne M. Leonard,[d] Denise C. Welsh,[d] Joseph J. Lynch[d] and Daniel R. McMasters[e]

[a]*Department of Medicinal Chemistry, Merck Research Laboratories, West Point, PA 19486, USA*
[b]*Department of Biological Chemistry, Merck Research Laboratories, West Point, PA 19486, USA*
[c]*Department of Preclinical Drug Metabolism, Merck Research Laboratories, West Point, PA 19486, USA*
[d]*Department of Pharmacology, Merck Research Laboratories, West Point, PA 19486, USA*
[e]*Department of Molecular Systems, Merck Research Laboratories, West Point, PA 19486, USA*

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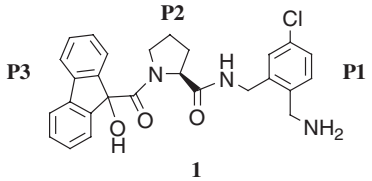
Abstract—Modification of lead compound **1** by reducing lipophilicity in the P3 group produced a series of low molecular weight thrombin inhibitors with excellent potency in functional assays, metabolic stability, and oral bioavailability. These modifications led to the identification of two optimized compounds, **14** and **16**.

1. Introduction

The serine protease, thrombin, occupies a central role in the coagulation cascade. The primary actions of thrombin are the activation of platelets and cleavage of fibrinogen to fibrin, which together constitute the primary components of a vascular thrombus. Inhibitors of thrombin have long been recognized as potential therapeutic agents for the treatment of a variety of thrombotic disorders, for example, deep vein thrombosis, pulmonary embolism, and thromboembolic stroke. Indeed, intravenous and oral thrombin inhibitors have shown promising results in human clinical trials[1,2] resulting in the recent approval of oral ximelagatran.[3] Our goal has been to identify a thrombin inhibitor with a convenient dosing regimen, that is, once or twice daily oral dosing, predictable pharmacokinetics, and no food or drug–drug interactions. Such a compound would potentially overcome the limitations associated with the two most widely employed antithrombotic agents, low molecular weight heparin (parenteral administration only), and warfarin (slow onset of action, chronic patient monitoring required).

An interesting thrombin inhibitor lead from our laboratories, **1**, resulted by combining an optimized P3–P2 portion of a compound from a proline P2-based series of thrombin inhibitors[4] with a recently discovered potency-enhancing P1 group.[5] Compound **1** was found to have good potency as a thrombin inhibitor in an isolated enzyme assay ($K_i = 0.5$ nM), good potency in a coagulation assay in human plasma ($2\times$APTT $= 0.37$ μM), and an extremely favorable pharmacokinetic profile in dogs ($F = 39\%$, iv $t_{1/2} = 13$ h). A shortcoming with **1**, however, was its poor efficacy in a $FeCl_3$-induced arterial thrombosis model in rats. The lack of efficacy in this animal model was attributed to the high plasma protein binding of **1**.



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* Corresponding author. Tel.: +1-215-652-9102; fax: +1-215-652-3971; e-mail: matthew_morrissette@merck.com

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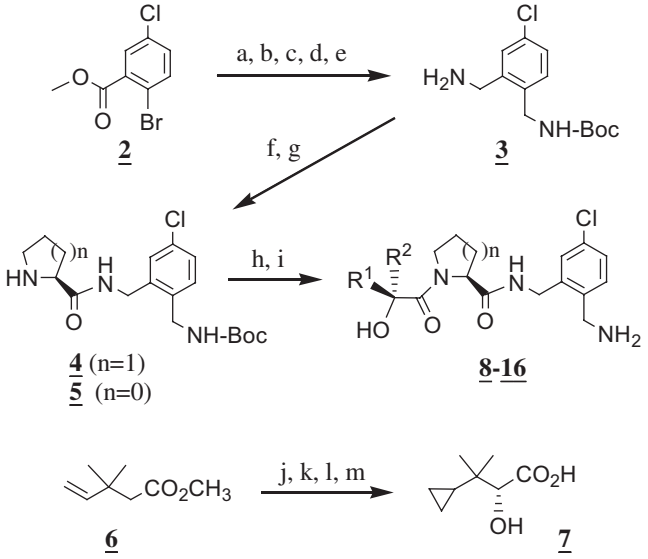


Scheme 1. Reagents and conditions: (a) $Zn(CN)_2$, $Pd(PPh_3)_4$, DMF, 60 °C; (b) $LiAlH_4$, THF; (c) Boc_2O, THF; (d) DPPA, toluene; (e) PPh_3, H_2O, THF; (f) Fmoc-amino acid, EDCI, HOBT, *i*-Pr_2NEt, DMF; (g) piperidine, DMF; (h) hydroxy acid, EDCI, HOBT, *i*-Pr_2NEt, DMF; (i) HCl gas, EtOAc, 0 °C; (j) CH_2N_2, cat. $Pd(OAc)_2$, ether; (k) LDA, THF, −78 °C, Davis oxaziridine; (l) NaOH, H_2O, MeOH; (m) chiral HPLC separation.

In this report we describe an approach to decreasing plasma protein binding, which involves reducing the size of the lipophilic fluorenyl P3 group in **1**. This approach has resulted in the production of potent, low molecular weight thrombin inhibitors with excellent efficacy, selectivity, metabolic stability, and pharmacokinetic properties.

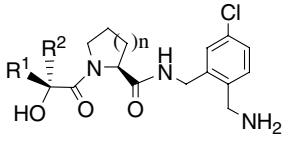
2. Chemical synthesis

Methyl 2-bromo-5-chloro-benzoate **2** was converted to 2-(N-Boc-aminomethyl)-5-chlorobenzylamine **3**[5,6] in five steps, and standard peptide coupling and deprotection reactions with Fmoc-L-proline or Fmoc-L-azetidine carboxylic acid provided multi-gram quantities of P2–P1 intermediates **4** and **5**, respectively (see Scheme 1). Standard peptide coupling and deprotection procedures using **4** and **5** and commercially available hydroxy acids provided compounds **1** and **8**–**15** in Table 1.[6] The hydroxy acid used for the synthesis of compound **16** in Table 1 was obtained from methyl 3,3-dimethyl-4-pentenoate **6**. The olefin in **6** was cyclopropanated, and this product was hydroxylated using the camphor-based oxaziridine reported by Davis et al.[7] The resulting 4:1 mixture of *R* and *S* hydroxy ester enantiomers was hydrolyzed and the hydroxy acids were separated by chiral HPLC to give **7**. Standard peptide coupling of hydroxy acid **7** and amine **5** followed by deprotection provided **16**. All final compounds were purified by reverse phase HPLC and isolated as amorphous TFA salts with purities greater than 95%.

3. Biological assays

Inhibition constants (K_i values) were determined for test compounds in an assay using human derived thrombin, trypsin, urokinase, plasmin, kallikrein, t-PA, and chymotrypsin with the corresponding fluorescent or chromogenic substrate under steady state conditions as previously described.[8,9] The concentration of test compound required to double the activated partial

Table 1. Biological results for compounds **1**, **8**–**16**



Compd	*n*	R^1	R^2	K_i[a] (nM)	2×APTT[b] (μM)	Plasma protein binding (% free)		In vivo rat[c] (occlusions)	Dog 1 mg/kg po[d]	
						Human	Rat		AUC (μM h)	$t_{1/2}$ (h)
1	1	—	—	0.50	0.37	1	9	4/5	30	13
8	1	Me	Ph	4.7	0.47				21	3.1
9	1	Ph	Me	2.7	0.33	15	24	1/6	14	3.4
10	1	H	Ph	0.90	0.14				1.4	2.0
11	1	Ph	H	80						
12	1	H	*c*-Hex	0.27	0.15	4	36	0/6	1.7	2.0
13	1	H	*i*-Pr	5.6	0.37	63	60	2/6	4.9	4.2
14	1	H	*t*-Bu	2.1	0.23	24	54	0/6	10	5.4
15	0	H	*t*-Bu	5.0	0.37	21	26	0/5	3.5	3.4
16	0	H	–C(Me)$_2$*c*–Pr	3.7	0.28	11	17	0/5	11	4.7

[a] Inhibition constants for human thrombin.
[b] Concentration of test compound required for doubling the activated partial thromboplastin time in human plasma.
[c] Test compounds administered at 10 μg/kg/min prior to $FeCl_3$-induced clot formation on the carotid artery, results are expressed as number of vessel occlusions in five or six animals.
[d] Test compounds dosed as TFA salts in 1% methocel suspension, results are expressed as area under the concentration versus time curve (AUC) and plasma half life ($t_{1/2}$).

thromboplastin time of human plasma, the 2×APTT value, was determined under conditions involving a threefold dilution of human plasma as previously reported.[9] The antithrombotic activities of intravenously administered test compounds were determined in an anesthetized rat model of topical $FeCl_3$-induced arterial thrombosis.[10] Test compounds were administered by intravenous infusion at a dose of 10 μg/kg/min for 180 min, $FeCl_3$ was applied to the carotid artery at 120 min post dose, and carotid artery blood flow was monitored for 60 min. Results are expressed as number of animals, which had occluded vessels ($n = 5$ or 6). For plasma pharmacokinetic assays, amorphous TFA salts of test compounds were dosed orally in 1% methocel solution to beagle dogs ($n = 2$) at 1 mg/kg. Blood samples were obtained over 8 h post dose, and a final blood sample was taken at 24 h. At each time point, test compound was isolated from the plasma fraction by solid phase extraction and quantified by LC–MS/MS against a standard curve. Area under the curve (AUC) and plasma half life ($t_{1/2}$) were determined from analysis of the concentration versus time curve. Plasma protein binding was determined as previously described.[11]

4. Results and discussion

The first two analogs of **1** that were synthesized in order to reduce lipophilicity in P3 were the phenylhydroxypropionamide diastereomers **8** and **9** (see Table 1). Interestingly, diastereomers **8** and **9** were of similar potency in the enzyme assay, with the *S* isomer **9** being slightly more potent. This is in contrast to previously established structure–activity relationships for P3 diastereomers in which one of R^1 and R^2 is hydrogen. For these compounds, there has generally been observed a large difference in potency between *R* and *S* isomers with the *S* isomer being less potent.[12] Indeed, diastereomers **10** and **11** with methyl replaced by hydrogen were found to exhibit the usual profile in the enzyme assay with the *S* isomer **11** being significantly less potent. Molecular modeling provided a rationale for these results. The lowest energy conformation[13] of **11** places the P3 phenyl group away from the S3 pocket of the enzyme. Rotating about the C═O, C_α carbon–carbon bond in the P3 group of **11** to bring the phenyl group into the proper orientation for binding to the S3 pocket raises the energy by 3.6 kcal/mol. The lowest energy conformation of **9**, on the other hand, places the P3 phenyl group in an ideal orientation for binding to the S3 pocket of the enzyme. Interestingly, however, this low energy conformation of **9** also places the hydroxyl group out toward solvent and unable to hydrogen bond to Gly-216 such as is the usual paradigm for P3 group interactions in peptide-based thrombin inhibitors. Corroboration of this proposed binding mode of **9** awaits verification by X-ray crystallographic analysis of the enzyme–inhibitor complex. Figure 1 shows an overlay of **9** and **11** in their low energy conformations docked into the enzyme active site.

As was anticipated, plasma protein binding experiments with **9** showed a large increase in the free fraction compared to lead compound **1** in both human and rat plasma. The increased free fraction of **9** in human plasma explains the observation that, even though **9** is five times less potent than **1** in the enzyme inhibition assay, the two compounds are very close in potency in the 2×APTT assay, which is run in the presence of human plasma. Also, the increased free fraction of **9** compared to **1** in rat plasma is presumably responsible for the improved performance of **9** in the in vivo thrombosis model in rats. Both **8** and **9** were well absorbed after a 1 mg/kg oral dose in dogs, but the plasma half life of both compounds was significantly diminished relative to **1**. Absorption and plasma half life in dogs continued to diminish with the phenylhydroxyacetamide P3 analog **10**, so attention was turned to investigating P3 analogs containing nonaromatic substituents. The *c*-hexyl derivative **12** proved to be very potent in both the enzyme and 2×APTT assays, with full efficacy in the in vivo thrombosis model in rats. The pharmacokinetic behavior of **12** in dogs, however, was not improved over that of **10**. Additional studies with **12** had shown that it was rapidly degraded in the presence of dog hepatic microsomal preparations, with oxidation occurring predominantly on the *c*-hexyl ring.

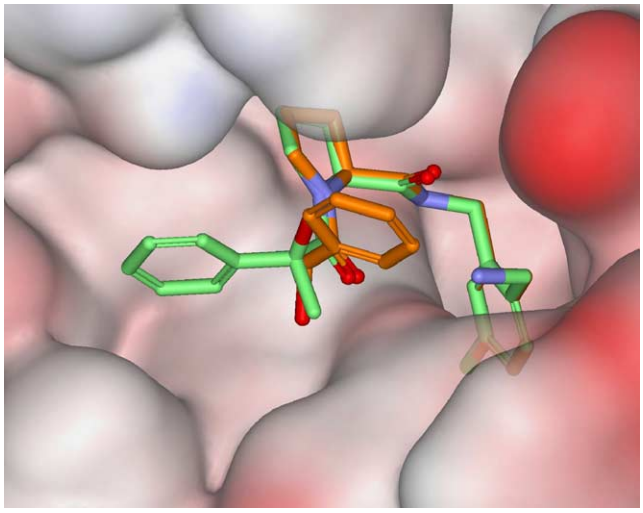


Figure 1. Compounds **9** (green) and **11** (orange) docked in the thrombin active site, shown as a transparent surface.

Removing potential sites of metabolism in **12** by truncating the *c*-hexyl group to an *i*-propyl group (**13**) indeed had a beneficial effect on dog hepatic microsome stability and also improved the oral pharmacokinetic profile in dogs. Potency and in vivo efficacy in rats, however, were reduced with the smaller P3 side chain in **13**. Increasing the size of P3 side chain to a *t*-butyl group provided a compound (**14**), which was found to possess a very favorable combination of properties: good potency in the 2×APTT assay, full efficacy in the in vivo thrombosis model in rats, little inhibition of several other serine proteases ($K_i = 5.9$ μM against trypsin; $K_i > 10$ μM against urokinase, plasmin, kallikrein, t-PA, chymotrypsin), oral bioavailability in three species with a good plasma half life (dog: $F = 81\%$, iv $t_{1/2} = 3.9$ h; rhesus monkey: $F = 46\%$, iv $t_{1/2} = 3.5$ h; rat: $F = 37\%$,

iv $t_{1/2} = 2.0$ h), and no inhibition of any of the major human cytochrome P450 isozymes at concentrations up to 100 μM. The potency of **14** in the isolated enzyme and 2×APTT assays compares very favorably with that of melagatran, the active form of the double prodrug ximelagatran,[3] measured in our assays ($K_i = 1.1$ nM, 2×APTT = 0.28 μM).

In vitro metabolism studies with **14** using human hepatic microsomes had shown a small amount of oxidative metabolism involving the P2 proline ring and the *t*-butyl side chain in P3. To address the former issue, the azetidine P2 analog **15** was investigated. Indeed, in vitro metabolism studies with **15** indicated that no metabolism had occurred on the azetidine ring. However, the smaller azetidine ring did result in a loss of potency. Potency could be recovered by increasing the size of the P3 side chain. For example, **16**, the analog of **15** in which one of the methyl groups of the *t*-butyl P3 side chain is replaced with a *c*-propyl group, had favorable potency and oral pharmacokinetic properties in dogs similar to those of **14**. Additionally, very little oxidative metabolism of **16** was observed in vitro using human hepatic microsomes.

5. Conclusion

In summary, reducing the size of the lipophilic P3 fluorenyl group in lead compound **1** gave a series of potent, low molecular weight thrombin inhibitors, which had reduced plasma protein binding and improved antithrombotic activity in rats. Compound **14** was found to possess an excellent combination of properties including high 2×APTT potency, high selectivity for inhibiting thrombin versus a panel of other serine proteases, and good pharmacokinetic properties in three animal species. Identification of sites of metabolism in **14** led to the design of **16**, which was found to possess very good in vitro metabolic stability as well as good potency and pharmacokinetic properties. Compounds such as **14** and **16** offer significant potential as drug development candidates for the treatment of various thrombotic disorders.

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