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Benzylamine-based Selective and Orally Bioavailable Inhibitors of Thrombin

Koo Lee,* Won-Hyuk Jung, Cheol Won Park, Chang Yong Hong, In Chul Kim, Sangsoo Kim, Yeong Soo Oh, O Hwan Kwon, Sung-Hack Lee, Hee Dong Park, Sang Woong Kim, Yong Hee Lee, and Yung Joon Yoo

Biotech Research Institute, LG Chemical Ltd./Research Park, P.O. Box 61 Yu Sung Science Town, Taejon 305-380, Korea

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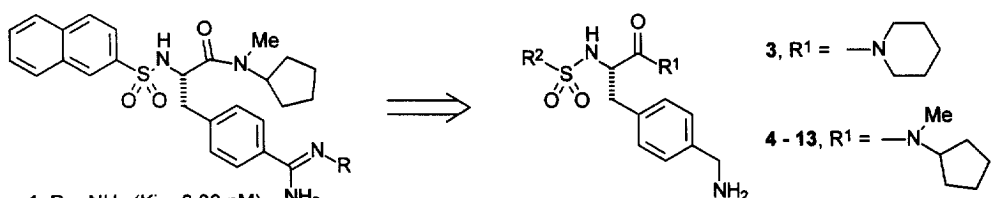
Abstract: A series of *p*-aminomethylphenylalanine derivatives were investigated as novel thrombin inhibitors. This study led to potent inhibitors of thrombin (Ki up to 3.3 nM) that are trypsin-selective, highly orally bioavailable in rats, and highly permeable across Caco-2 cells. The P1 benzylamine binding mode in the thrombin active site was identified by X-ray crystallographic analysis.

Thrombin is a critical mediator in thrombosis, and its inhibition has thus become a major therapeutic target in the treatment of cardiovascular diseases such as deep vein thrombosis, myocardial infarction, and pulmonary embolism.[1] While numerous small molecule inhibitors of thrombin have been discovered,[2] most of those agents suffer from lack of oral bioavailability. It is, therefore, clear that next generation thrombin inhibitors must possess oral bioavailability in order to be more clinically useful anticoagulants.[3]

The poor oral bioavailability of thrombin inhibitors is often associated with the highly basic P1 functionalities such as guanidine and amidine.[4] Recently, we identified compound **1** (LB30057) as a potent and orally bioavailable thrombin inhibitor.[5] We presume that the good oral bioavailability of **1** is to some extent benefited by the less basic amidrazone function (pKa ~ 8.9); the corresponding benzamidine analog **2** proved poorly bioavailable.[6] As our continuing efforts towards developing new class of oral thrombin inhibitors, we investigated the benzylamine group as a P1 replacement which has a suitable pKa (9.35[7]). Although some compounds in this class were studied by Sturzebecher et al., the potency was very limited: the best inhibitor was reported to have a Ki of 3.1 μM (i.e. **3**, R^2 = 2-naphthyl).[8] Furthermore, oral activities of this type of compounds have not been described to date. In this report we describe the discovery of benzylamine-based thrombin inhibitors that are selective and orally active.



1, R = NH_2 (Ki = 0.38 nM)
2, R = H (Ki = 6.8 nM)

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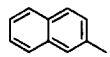
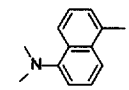
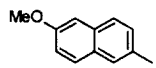
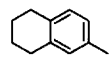
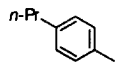
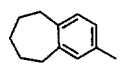
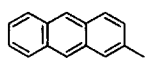
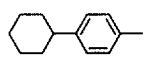
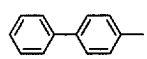
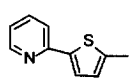
Table 1. In vitro and in vivo activities of *p*-aminomethylphenylalaine derivatives[9]

Compound	R^2	Ki (thrombin[a], nM)	Ki (trypsin[b], nM)	Thrombus inhibition effect in rats (%)[c]
4		18.3	13,600	17.6
5		10.9	17,100	35.5
6		6.6	14,200	44.1
7		10.1	9,800	16.4
8		9.6	9,040	ND
9		3.3	6,300	39.4
10		5.7	3,300	45.4
11		20.1	4,800	23.8
12		60.5	ND	ND
13		107	ND	ND

[a] human thrombin [b] bovine trypsin [c] thromboplastin-induced venous thrombosis[13] (5 mg/kg iv bolus)

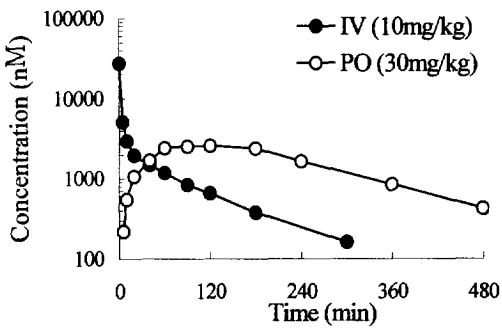
N,N-Cyclopentylmethylamide derivative (**4**) was found to be optimal in the set of amide derivatives in our preliminary SAR study of this series by the resin-bound synthesis.[10] Compound **4**, when reprepared in pure form, exhibited good thrombin inhibitory activity with a Ki of 18 nM. This compound also showed good selectivity versus trypsin. This finding led us to further explore several sulfonamide aryl moieties which were comparable or superior in thrombin affinity to the 2-naphthyl group in the study of benzamidrazone series.[11] We chose ten replacements of the naphthyl group as listed in Table 1.[12] Compounds **5-10** showed increased potency over **4** (two to six fold) with the best compound displaying a Ki of 3.3 nM. Biaryl replacements (compounds **12, 13**) resulted in significant losses of potency, while *p*-cyclohexylphenyl moiety (**11**) was fairly tolerated. Presumably, the biaryl groups of this series reach deeper in the D-pocket of the thrombin active site than that of the amidrazone series, causing less favorable binding affinity as can be explained by comparing the structures of **1** and **6** bound to thrombin (*vide infra*). In the evaluation for trypsin-selectivity, these compounds displayed more than sufficient levels ($Ki_{thr}/Ki_{tryp} > 500$).

The compounds were evaluated for their in vivo antithrombotic activity in the rat thromboplastin-induced model of venous thrombosis (see Table 1).[13] At an iv bolus dose of 5 mg/kg, the potent inhibitors **6**, **9**, and **10** were 39 to 45% effective in inhibiting thrombus formation, whereas the inhibitor **4** displayed significantly reduced efficacy. However, this series of compounds demonstrated generally good pharmacokinetic profile in the oral absorption studies in rats.[14] As illustrated in Table 2, compounds **5**, **6**, and **9** show high peak blood concentrations and good oral bioavailability (77% for **6**). It is also noteworthy that these compounds showed long duration in blood after absorption: as exemplified by compound **6** in Figure 1, the blood concentration after 6 hours was about a half C_{max}.

Table 2. C_{max} and T_{max} for **5**, **6**, and **9** after oral administrations to rats (30 mg/kg, n = 3-4).

Parameters	**5**	**6**	**9**
C_{max}(μM)	3.3	2.8	3.5
T_{max}(min)	96	140	80

Figure 1. Blood concentrations of compound **6** in rats



Although the thrombin inhibitors herein exhibited somewhat moderate in vivo activity in the iv models, such promising pharmacokinetic results prompted us to examine their oral efficacy. We anticipated that the limited efficacy might be overcome by the high absorption and the sufficient blood concentration maintained thereof. Thus compound **6** was chosen and evaluated for its oral efficacy on the rat venous thrombosis (n = 4). The thrombi measurement was carried out at two time points where high blood concentrations persisted. At 2 hours after administration (50 mg/kg po), compound **6** inhibited the thrombus formation by 54%; at later 4 hours, indeed nearly equipotent inhibition effect was observed (47%). These results are remarkable in terms of long duration of action.

In contrast to the rat results, the dog pharmacokinetic evaluation with those three compounds resulted in low oral bioavailability (10-15%); the blood concentration in this species was significantly lower than that attained in rats. It is presumable that the low absorption in dogs is largely due to the relatively low membrane permeability compared to that of rats.[15] In order to further understand the absorption behavior of this class of

compounds, we performed in vitro permeation experiment using a human Caco-2 cell monolayer.[16] Structurally related compounds **1** and **2** were also used for comparison. Surprisingly, the benzylamine **4** exhibited excellent apparent permeability coefficient (P_{app}) which is an order of magnitude higher than that of **1** (see Table 3). Similar values of P_{app} were also obtained for other compounds of this series including **6** while the amidine counterpart **2** showed poor permeability.[17] Since the cell-line used in this system is human-originated, these results might justify the pharmacokinetic evaluation in humans despite the species difference in absorption.[18]

Table 3. Apparent permeability coefficients for **1**, **2**, **4**, and **6** across Caco-2 cell monolayer (n = 3)

	1	**2**	**4**	**6**
P_{app} (nm/sec)	17	2	110	107

The binding modes of the benzylamine inhibitors were determined by X-ray crystallography. The crystals of **6** bound to the human α-thrombin-hirugen complex were prepared and used in X-ray data collection as described previously.[5b] The crystal structure was determined at 2.0 Å with an R factor of 0.19. A simple superposition of the structures of **6** and **1** in the active site of thrombin is shown in Figure 2. The overall binding mode of **6** is very similar to that of **1** as anticipated by modeling. The biggest difference in the binding modes of **6** and **1** lies in the P1 specificity pocket. Unlike the amidrazone group of **1** which interacts directly with the carboxylate of Asp 189, the benzylic amine of **6** has no direct interaction with Asp 189. Instead, it forms a hydrogen bonding network with C=O of Ser 214 and two ordered water molecules, each N···O distance being 3.1, 2.7 and 2.7 Å. One of the water molecules has additional hydrogen bonds to Asp 189 carboxylate

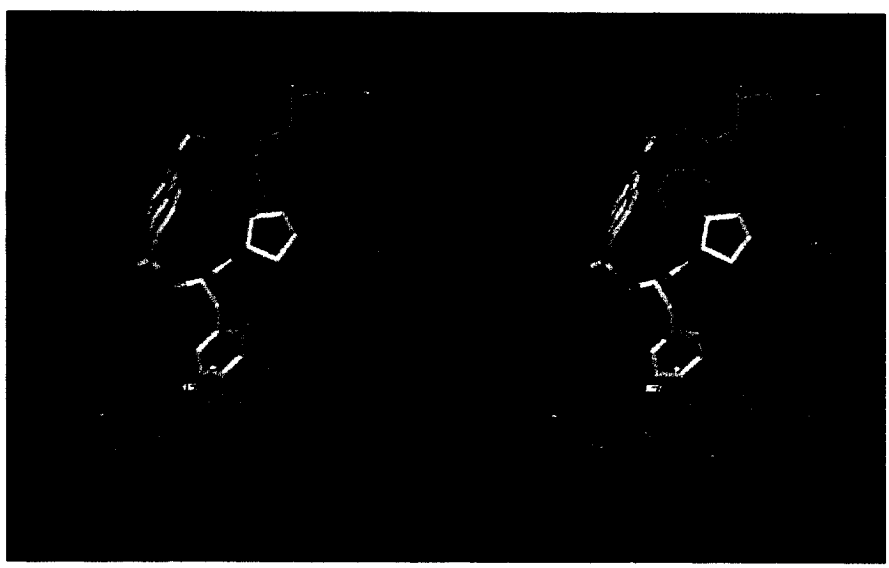


Figure 2. Stereoview of inhibitors **6** (thick) and **1** (thin, purple) bound in the thrombin active site.

(2.9 Å) and C=O of Gly 219 (3.1 Å). The other water molecule occupies the position where the amidrazonyl amine of **1** was located, and forms an additional hydrogen bond with C=O of Phe 227 (3.1 Å). The benzylic amine is completely out of the phenylene plane with a dihedral angle of 90°, causing overall shape of the benzylamine moiety less planar compared to that of the benzamidrazone of **1**. Consequently, the benzylamine moiety of **6** is displaced by about 0.5 Å out of the P1 pocket compared to the benzamidrazone of **1**. Despite of this displacement, the hydrogen bonds with Gly 216 backbone atoms are maintained and the P-pocket moiety, N,N-cyclopentylmethylamide, is anchored at the same position. However, the 6-methoxynaphthyl group of **6** is shifted slightly deep inside the D-pocket compared to the naphthyl group of **1**.

In summary, we have prepared and evaluated a series of thrombin inhibitors that incorporated *p*-benzylamine element at the P1 position. These compounds displayed good trypsin-selectivity and good absorption behavior in rats although their antithrombotic efficacy needs to be improved by further structural optimization. Especially noteworthy are their long duration in blood in the rat absorption study, as well as high permeability in the Caco-2 cell system. In addition, these inhibitors (i.e. **6**) demonstrated long duration of action in the rat models for oral efficacy. Since long-lasting oral agents are desirable in the development of future antithrombotics, this finding is an important step in our continuing search for next generation of thrombin inhibitors. Further SAR and pharmacokinetic studies with this series of thrombin inhibitors are in progress; the results will be published in due course.

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References and Notes.

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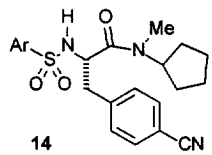
12. The compounds in Table 1 were prepared from the corresponding *p*-cyanophenylalanine sulfonamide derivatives (general structure **14**[11]), the same intermediates as used in the synthesis of a series of benzamidrazone compounds including **1**. Hydrogenation of **14** at 60 psi in the presence of methanol and a small amount of concentrated HCl afforded the desired targets as acid salts within 24 h in generally good yields. All of the compounds were purified by HPLC.



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14. Male Sprague-Dawley rats (200-300 g) were used; for the oral studies the rats were fasted for 18 h. The femoral artery was cannulated with polyethylene tubing (PE-50) under light ether anesthesia. For the iv kinetic studies the femoral vein was additionally cannulated. After complete recovery from anesthesia, compounds were administered orally or intravenously. Approximately 250 μL aliquots of blood were withdrawn via the femoral artery into heparinized tube (25 U/ml) and the collected blood samples were immediately treated with methanol and zinc sulfate for HPLC analysis.
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