Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 631–634

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

DISCOVERY OF LB30057, A BENZAMIDRAZONE-BASED SELECTIVE ORAL THROMBIN INHIBITOR

Yeong Soo Oh*, Mikyung Yun, Sang Yeul Hwang, Seongwon Hong, Youseung Shin, Koo Lee, Kyung Hee Yoon, Yung Joon Yoo, Dong Soo Kim, Sun Hwa Lee, Yong Hee Lee, Hee Dong Park, Chang Ho Lee, Sang Koo Lee, and Sangsoo Kim*

Biotech Research Institute, LG Chemical LTD/Research Park, P.O.Box 61 Yu Sung, Taejon 305-600, KOREA

Received 26 August 1997; accepted 5 February 1998

Abstract. Systematic variation of the so-called P-pocket moiety of benzamidrazone-based selective thrombin inhibitors led to the discovery of LB30057. It is potent (Ki = 0.38 nM for human thrombin), selective (Ki = 3290 nM for bovine trypsin), and orally bioavailable (58% oral bioavailability in dogs). LB30057 was efficacious in thrombosis animal models.

Pathological clot formation is the major cause of serious diseases such as deep vein thrombosis, pulmonary embolism, myocardial infarction and stroke. Thrombin, a potent mediator of thrombosis, is one of the targets for the antithrombotic therapeutics.[1] Recent publications on thrombin inhibitor discovery dealt with issues like oral bioavailability, selectivity, and potency among others.[2] Previously we reported a novel strategy of designing highly selective, potent thrombin inhibitors.[3] The benzamidrazone compound **1** was the

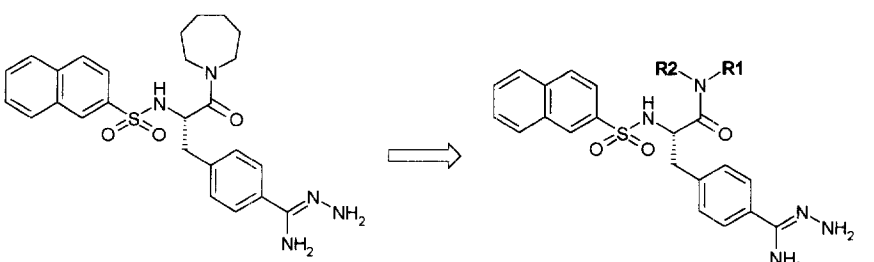

Figure 1. Structures of thrombin inhibitors.

most potent among the series (Ki = 1.47 nM for bovine thrombin) but its limited water solubility (<< 1 mg/ml) hampered further development. Here we report the replacement of the hexamethyleneimine moiety of **1** with various acyclic secondary amines (see Figure 1). If the former is replaced with primary amines, the resulting amide bond will have *trans* conformation only. On the other hand, secondary amines offer advantage of exploring both *cis* and *trans* conformations. Using the synthetic scheme previously reported,[3] we prepared the compounds listed in Table 1.

PII: S0960-894X(98)00079-1

Table 1. Inhibitory effect[a] of thrombin inhibitors

Compound	R1	R2	Ki (μM)
2	Methyl	Cyclopropyl	0.247
3	Methyl	n-Butyl	0.041
4	Methyl	Cyclopentyl	0.0011
5	Methyl	Cyclohexyl	0.152
6	Ethyl	Cyclopentyl	0.004
7	Propyl	Cyclopentyl	0.093
8	n-Butyl	Cyclopentyl	0.231
9	Hydroxyl	Cyclopentyl	0.367
10	2-Hydroxyethyl	Cyclopentyl	7.040

[a]The inhibitory activity against bovine thrombin was measured according to the previously reported procedure.[4]

It is clear that cyclopentyl group is the optimum in size at **R2** (Compounds **2-5**). Substitution at **R1** with alkyl groups bigger than methyl resulted in substantial increase in Ki values (Compounds **6-8**). Compared to their isosteric alkyl counterparts, hydrophilic groups at **R1** (*e.g.* **4** vs. **9** and **7** vs. **10**) have deleterious effect on potency. In this series, the best combination was achieved with methyl and cyclopentyl moieties at **R1** and **R2**, respectively (**4**, LB30057). LB30057 had excellent thrombin inhibitory activity (Ki = 1.1 nM and 0.38 nM against bovine and human thrombins, respectively) and excellent selectivity over other trypsin-like serine proteases (Ki = 47.9, 27.2, 1.1, and 3.2 μM against human plasmin, tPA, factor Xa, and bovine trypsin, respectively).[5] Interestingly, the water solubility of LB30057 (>> 10 mg/ml) was much higher than that of **1**. It is not clear how the structural difference between *N*-cyclopentyl-*N*-methylamide and hexamethyleneimide causes the large difference in water solubility.

In order to comprehend the structure-activity relation, the crystal structure of LB30057-thrombin complex was determined.[6] The structure of LB30057 bound at the active site of thrombin is shown in Figure 2. The naphthyl group in the D-pocket is found in close contact with the side chain of Ile 174 (closest approach 3.4Å). Other close contacts (under 3.7Å) are with the side chains of Trp 215 and Tyr 60A. The cyclopentyl group is in the *cis* position of the amide bond and makes tight van der Waals interaction with Trp 60D (3.6Å), Tyr 60A (3.7Å) and Lys 60F (3.8 Å). It appears that cyclopropyl group of **2** is too small to provide this kind of interaction, while cyclohexyl group of **5** would not fit into the pocket very well. The methyl group also contributes to the binding affinity considerably, through the hydrophobic interactions with Leu 99 (3.7Å) and His 57 (4.0Å). In contrast to the methyl group, hydrophilic groups were unfavorable substituents (Compounds

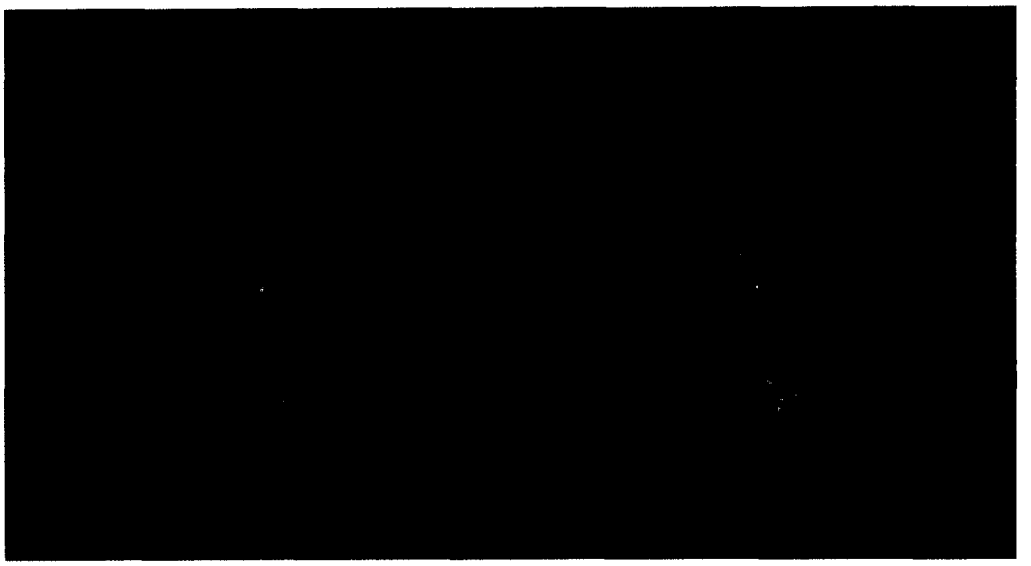

Figure 2. Stereoview of LB30057 (thick connections) bound to the active site of human thrombin (thin connections). The naphthyl group of LB30057 interacts with the D-pocket which is defined by the side chains of Trp 215, Leu 99, Tyr 60A and Trp 60D; the *N*-cyclopentyl-*N*-methyl moiety fits into the P-pocket which is defined by Leu 99, His 57, Tyr 60A, Trp 60D and Lys 60F.

9, 10). Thus, this crystal structure demonstrates that LB30057 efficiently interacts with active site of thrombin at D- and P-pockets and that the *N*-cyclopentyl-*N*-methylamide moiety is a good P-pocket binder.

The antithrombotic efficacy of LB30057 was examined in a rat venous thrombosis model: With 5 and 1 mg/kg intravenous bolus administrations, thromboplastin-induced thrombus formation was inhibited 90% and 50%, respectively.[7] In a dog arterial thrombosis model,[8] intravenous bolus administration of 10 mg/kg LB30057 resulted in 2-fold increase in mean occlusion time. The oral bioavailability of LB30057 was 58% with the corresponding elimination half-life of 112 min at 10 mg/ml dose in dogs.[9] LB30057, a potent, selective, and orally bioavailable thrombin inhibitor, is a promising antithrombotic agent and its further pharmacological evaluation is under way.

Acknowledgment

We thank Drs. J.M. Cho, Y.Z. Kim, and I.C. Kim for support and encouragement during this work. One of the author (S. K.) thanks Drs. Eunice E. Kim and C.Y. Hong for critical comments on the manuscript and the Korea Institute of Science and Technology - the System Engineering Research Institute (KIST-SERI) for the Cray time used in the crystal structure refinements.

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5. Details of the enzyme inhibition kinetics will be published elsewhere.
6. Human thrombin was purified essentially according to the procedure described by Fenton et al (Fenton, J.W. II; Fasco, M.J.; Stackron, A.B.; Aronson, D.L.; Young, A.M.; Finlayson, J.S. *J. Biol. Chem.* **1972**, *252*, 3587). Thrombin crystals grown in the presence of hirugen and a weak inhibitor (not described here) according to the procedure reported by Qiu et al (Qiu, X.; Yin, M.; Padmanabhan, K.P.; Krstenansky, J.L.; Tulinsky, A. *J. Biol. Chem.* **1993**, *268*, 20318), were soaked overnight in the buffer containing 2 mM LB30057. The completeness of inhibitor exchange was confirmed later from the electron density. X-ray diffraction data up to 2Å resolution were collected using MacScience DIP-2020 imaging plate system, with mirror-focused CuK α X-ray radiation generated by a MacScience MX06HF rotating anode generator operating at 50kV, 90mA. The space group was C2 with cell dimensions of a = 70.9, b = 72.0, c = 73.3Å and β = 100.8°. A set of 60 frames were collected from a crystal with an oscillation ange of 1.5°, and the exposure time of 50 min. A total of 61,872 diffraction spots were measured and averaged to 22,879 unique reflections with an R_{sym} = 6.5% using the Denzo package (Z. Otwinowski and W. Minor). The protein model was taken from the entry 1HGT of the Protein Data Bank (PDB) and refined using a simulated annealing protocol (Brünger, A.T.; Kuriyan, K.; Karplus, M. *Science* **1987**, *235*, 458). The inhibitor molecule was built into the subsequent difference density and the whole complex including 234 water molecules were refined to an R = 19.9%. The r.m.s. deviations of bond distances and angles from ideal values were 0.010Å and 1.9°, respectively.
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9. LB30057 was administered via the cephalic vein of left leg for i.v. study and administered via gavage for oral study to determine its pharmacokinetics in Beagle dogs. The blood was withdrawn via the cephalic vein of right leg (i.v. only) up to 720 min. The blood samples were, then, deproteinized by mixing 1.5 volume of methanol and 0.5 volume of 10% $ZnSO_4$, and the supernatant was quantitated using HPLC on a reversed-phase column at UV 231 nm.