Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 1, pp. 67-72, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(96)00583-5

SYNTHESIS, EVALUATION, AND CRYSTALLOGRAPHIC ANALYSIS OF L-371,912: A POTENT AND SELECTIVE ACTIVE-SITE THROMBIN INHIBITOR

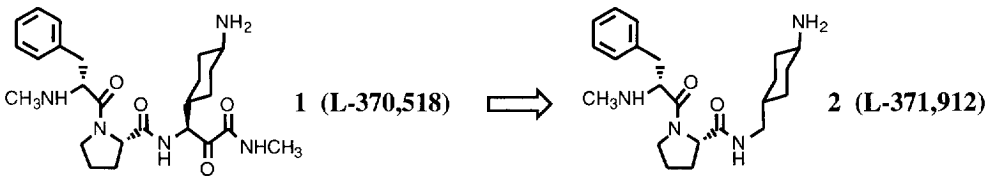
Terry A. Lyle,*[b] Zhongguo Chen*[a] Sandra D. Appleby,[d] Roger M. Freidinger,[b] Stephen J. Gardell,[a] S. Dale Lewis,[a] Ying Li,[a] Elizabeth A. Lyle,[d] Joseph J. Lynch Jr.,[d] Anne M. Mulichak,[a] Assunta S. Ng,[a] Adel M. Naylor-Olsen,[c] and William M. Sanders[b]

Departments of [a]Biological Chemistry, [b]Medicinal Chemistry, [c]Molecular Design & Diversity, and [d]Pharmacology, Merck Research Laboratories, West Point, PA 19486 U.S.A.

Abstract: Removal of the β-ketoamide functionality from L-370,518 (K_i = 0.09 nM) provided a 5 nM K_i inhibitor of thrombin: L-371,912. Comparison of the enzyme-inhibitor crystal structures suggests a possible explanation for the relatively small change in affinity for thrombin. L-371,912 is selective for thrombin over related serine proteases and is efficacious in an animal model of arterial thrombosis.

From a survey of the recent literature, it is clear that the discovery of a highly selective, potent, and orally bioavailable inhibitor of the serine protease thrombin is a top priority for many research laboratories throughout the world. The many limitations of approved anticoagulants and those currently in clinical trials are major reasons to develop such an agent.[1] An ideal thombin inhibitor would provide predictable levels of anticoagulation when administered parenterally or orally, with minimal danger of bleeding or unrelated side effects. A highly selective inhibitor of thrombin that met these criteria would be an attractive candidate for drug development.

One of our initial objectives for an orally active thrombin inhibitor was to achieve as much selectivity as possible over trypsin, an important, closely related digestive enzyme. Recent work from these laboratories has described one approach to achieving substantial trypsin selectivity starting from traditional tripeptide inhibitors. The result of these efforts was the novel inhibitor L-370,518 (**1**) containing a *trans*-aminocyclohexylglycine ketoamide residue at the P1 position, traditionally occupied by a lysine or arginine.[2]



This compound is based upon the well-known D-Phe-Pro-Arg-H prototype in which the basic Arg residue binds to Asp189 in the S1 subsite, the proline ring sits in the S2 subsite, and the phenyl ring of the D-Phe residue occupies the P3 "aryl binding site" of the enzyme. A key interaction is the covalent bond between the electrophilic aldehyde and Ser195 of the enzyme. This type of covalent interaction is utilized in many potent inhibitors of serine proteases, and is commonly found in the D-Phe-Pro-Arg tripeptide-type thrombin inhibitors.[1a-c] Many other groups have been employed as electrophiles for the serine hydroxyl (e.g., chloromethyl ketones, α,α-difluoroketones, phosphinic acids, and boronic acids).[1] Compound **1** utilizes a primary amine as the basic group to interact with Asp189, and an α-ketoamide as the Ser195 electrophile. The side chain at the S1 site was designed

to take advantage of the difference in the steric and electrostatic environments of the "selectivity pockets" of thrombin and trypsin, resulting in an inhibitor with a K_i of 90 pM for thrombin, and 1.15 μM for trypsin.[2] This 13,000-fold improvement in selectivity over the corresponding acyclic analogs[3a,b] was an important step in our search for a clinical candidate.

Some aspects of the aminocyclohexylglycine ketoamide group of compound **1** were of concern, including stability, toxicity, and difficulty of synthesis. One approach to the latter problem was recently addressed in a publication from these laboratories.[4] We describe further progress in this area in the Chemistry section below. The other concerns stemmed from the presence of the relatively labile α-ketoamide electrophilic group, which has the potential to accelerate the rate of epimerization at the neighboring chiral center, as well as the possibility of making undesired covalent interactions with endogenous proteins and peptides. One possible solution to both of these problems would be to eliminate the α-ketoamide altogether. This somewhat radical transformation might be expected to dramatically alter the binding constant of the inhibitor because the key interaction with the active-site Ser 195 residue has been removed.

As a result of the improved synthetic route (Scheme 1) for compound **1**, it became possible to make the compound **2**, shown above, required to test this hypothesis. Comparison of the in vitro data for compound **2** with the α-ketoamide **1** provided a number of interesting results (Table 1).

Table 1

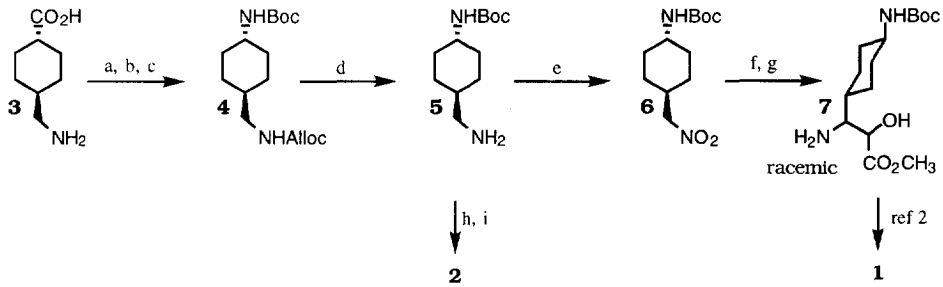
	1	**2**
K_i (nM) vs. thrombin (human)[5]	0.09 ± 0.02	5.0 ± 0.5
K_i (nM) vs. trypsin (bovine)[5]	1150 ± 300	11000 ± 3000
2 X APTT (μM)	2.0	1.1
# of occlusions @ 6 μg/kg/min iv	3/6	1/6

The 5 nM K_i of **2** for thrombin, although diminished with respect to **1**, was less than expected. A possible explanation for this value is optimization of nonbonded interactions, as elaborated in the Crystallography section below. The excellent selectivity over trypsin was maintained, presumably due to the steric and hydrophobic demands of the cyclohexyl ring, as previously suggested for **1**.[2] The concentration of **2** required to double the activated partial thromboplastin time (2 X APTT) in human plasma was found to be 1.1 μM. When **2** was evaluated in a rat ferric chloride model of arterial thrombosis,[3b,6] good efficacy (1/6 occlusions) was observed at an intravenous dose of 6 μg/kg/min.[7] The excellent efficacy observed for **2** as compared to **1** may be partially explained by the reduced 2 X APTT value and a modest increase in kon (data not shown). Oral bioavailability in several species was found to be 5-10%.[8]

Chemistry

In analyzing more efficient approaches to the synthesis of the novel *trans*--4-aminocyclohexylglycine ketoamide found in inhibitor **1**, two major synthetic challenges are apparent. The first is the *trans* relationship of the groups on the cyclohexyl ring, and the second is the creation of the somewhat labile α-ketoamide

functionality. One approach that effectively deals with both of these issues has been mentioned above,[4] and a second approach is described below in Scheme I. In order to address the problem of the *trans* stereochemistry on the cyclohexyl ring, the commercially available tranexamic acid **3** was used as the starting point for the synthesis. Protection of the amino group using allyl chloroformate followed by a Curtius rearrangement using diphenylphosphoryl azide and *t*-butoxide provided the differentially protected diamine **4**. Removal of the Alloc group in the presence of diethylamine and catalytic tetrakis(triphenylphosphine) palladium afforded the important intermediate **5**. Other nucleophilic reagents, such as dimedone and diethyl malonate, were tried in this reaction, but were less than satisfactory. Oxidation of the amine using 3-chloroperoxybenzoic acid gave the nitro compound **6**, which was purified by column chromatography. The key step in the synthesis involves a Henry-type reaction of **6** with methyl glyoxylate to generate a precursor to the α-ketoamide group as an α-hydroxyester. A variety of conditions for the condensation of **6** with methyl glyoxylate were attempted, but by far the most efficient was the use of potassium fluoride in anhydrous THF. These conditions provided the condensed nitroalcohol as a mixture of four diastereomers in good yield after column chromatography. Reduction of the nitro group was achieved by hydrogenation using a Raney nickel catalyst in methanol to afford the key intermediate **7**. Conversion of intermediate **7** to the ketoamide **1** was carried out with only minor modifications, as previously described.[2]



Scheme I. (a) allyl chloroformate, dioxane, 2 M NaOH, 97%; (b) DPPA, TEA, benzene, 45 °C; (c) 1 M LiOtBu/THF, 91%; (d) $(Ph_3)_4Pd$, DEA, THF, 92%; (e) mCPBA, benzene, Δ, 70%; (f) methyl glyoxylate, KF, THF, 70%; (g) Raney Ni, H_2, MeOH, 85%; (h) EDC, HOBT, BocN-Me-D-Phe-Pro, TEA, DMF; (i) HCl, EtOAc, 85%.

For the synthesis of **2**, the intermediate **5** was subjected to simple EDC-mediated coupling conditions with Boc-N-Me-D-Phe-Pro-OH, followed by Boc deprotection with HCl in ethyl acetate. The dihydrochloride of **2** was obtained as a colorless solid in good overall yield.

Crystallography

The complex crystals of human α-thrombin-hirugen with L-371,912 were prepared using procedures previously described.[9] X-ray diffraction data for the inhibitor complexes were collected using a Rigaku RAXIS II imaging plate system, with monochromatized CuKa X-ray radiation generated by a Rigaku RU 200 rotating anode generator operating at 50 kV, 100 mA. A single crystal was used for data collection, and the crystal diffracted to 1.8 Å resolution. The crystal was of space group C2, having cell parameters as a = 71.25 Å, b = 72.19 Å, c = 73.11 Å, and b = 100.85°. Data were collected in 60 frames with an oscillation angle of 2°, and the exposure time was 30 min per frame. The distance between crystal and imaging plate was 82 mm. The total data

consisted of 40,601 diffraction spots, including 23,015 unique reflections, and having a Rmerge of 7.87%. All data were processed and scaled using Rigaku RAXIS software.

The structure of the complex crystal was solved by the difference Fourier method, using as a starting model the atomic coordinates of a similar thrombin-inhibitor complex previously reported,[9] and after the inhibitor and nearby solvent molecules had been removed from the thrombin active-site region. All calculations for electron density maps and structure refinement were made employing the XPLOR program package,[10] and using only data at 6.0 to 1.8 Å resolution having intensities greater than a 2σ limit. Well-defined and continuous electron density in the active sites was found in both $2F_o$-F_c and F_o-F_c maps that clearly defined the location of the inhibitor in the complex. Unlike the map of the complex of thrombin-L-370,518 that showed a continuous electron density map between the inhibitor and Oγ Ser195 of the enzyme, the discontinuity of the electron density at this region in $2F_o$-F_c electron density map of thrombin-L-371,912 indicated there was no covalent bond between this inhibitor and Oγ Ser195. Inhibitors were built in the binding sites with the program CHAIN[11] on a SILICON Graphics Workstation. Most solvent molecules from the initial starting structure were also observed in the electron density maps. As the refinement progressed, additional water molecules were gradually added, based on the appearance of consistent peaks above a 2.5σ cutoff in both $2F_o$-F_c and F_o-F_c maps, which were within reasonable hydrogen-bonding distance and geometry to an appropriate atom in the thrombin complex. The final refined structure of the thrombin-hirugen-L-371,912 complex included 199 water molecules, and the R-factor was 17.2%. The root mean square deviation from ideal bond length and bond angles were 0.014 Å and 2.9°, respectively.[12]

The overlap of compounds L-371,912 and L-370,518, together with the surrounding residues of thrombin in the crystal structures are shown in Figure 1. The overall conformation of these two bound inhibitors is quite similar. Three major hydrogen bonds between the inhibitors and the backbone of the thrombin enzyme (O Ser214, N Gly216, and O Gly216) are inferred. Because there is no covalent bond between L-371,912 and the enzyme, the whole inhibitor shifts toward the P3 site, as can be seen in Figure 1. With a ~0.57 Å shift of the Cα atoms of the D-Phe group, the phenyl ring of L-371,912 sits almost in the same plane as that of L-370,518 in the S3 subsite. The entire proline residue in the S2 subsite has a global shift of 0.54 Å. The most noteworthy shift is found in the P1 group. After elimination of the strong tension caused by the covalent bond , the Cα atom of the L-371,912 P1 group shifts 1.22 Å away from the Cα atom in the thrombin-L-370,518 complex. Instead of inserting deeper in the specificity pocket to make a stronger electrostatic interaction with Asp189 of the thrombin enzyme, the "tension-free" aminocyclohexyl group stays at the same height in the specificity pocket and rotates ~140° around the Cα-Cβ axis of the P3 group. The amine nitrogen atom of the P3 group of L-371,912 remains in a similar position as in the L-370,518 complex. Four possible hydrogen bonds between the protonated amino group N3 and the thrombin enzyme can exist in both complex structures, and are listed in Table 2. In compensating for the shift of the aminocyclohexyl group in the S1 subsite, the residue Gly216 that forms part of the specificity pocket shifts 0.63 Å toward the S3 subsite. The Glu192 side chain of the thrombin-L-371,912 complex adjusts itself by rotating 90° around its Cα-Cβ bond to maximize the hydrophobic interaction with the cyclohexane ring.

Figure 1: Stereoview of the overlap of L-371,912 (yellow) and L-370,518 (green) in their complex structures. Surrounding thrombin residues are represented by thin lines with the same color code as their inhibitors.

Table 2: Possible Hydrogen Bonds Between Thrombin and Inhibitors L-371,912 and L-370,518.

Thrombin	**L-371,912**		**L-370,518**	
O Ser214	N ACH[a]	(2.95Å)	N ACH	(3.34Å)
N Gly216	O D-Phe	(2.73)	O D-Phe	(3.06)
O Gly216	N D-Phe	(3.11)	N D-Phe	(2.78)
Nε2 His57			O C1[d]	(2.59)
N Gly193			O C2[e]	(2.65)
N Ser195			O C2	(3.06)
O W757[f]			N2[b]	(2.88)
Nz Lys60F			O W757	(3.07)
O Ala190	N3	(2.82)	N3[c]	(2.87)
O Gly219	N3	(2.71)	N3	(2.83)
Oδ1 Asp189	N3	(3.22)	N3	(3.58)
O W 797	N3	(2.65)	N3	(2.71)
Oδ2 Asp189	O W797	(2.72)	O W797	(2.67)

[a]ACH = amino cyclohexane group. [b]N2 = nitrogen atom of the C-terminal methyl amide.
[c]N3 = nitrogen atom of the ACH group. [d]C1 = ketone carbonyl.
[e]C2 = C-terminal amide carbonyl. [f]W = H_2O.

Conclusion

Formal removal of the electrophilic ketoamide functionality from the thrombin inhibitor **1** affords L-371,912 (**2**), a structurally simple, low-molecular weight inhibitor of thrombin. The overall result is a compound that remains a potent thrombin inhibitor with 2200-fold selectivity over trypsin. In a rat model of arterial thrombosis, **2** was found to be efficacious at an intravenous dose of 6 μg/kg/min. Additionally, both the labile chiral center and the potential for unwanted covalent interactions with the reactive carbonyl group have been eliminated. Possible reasons for the less-than-expected difference in the K_i's between **1** and **2** are suggested in the analysis of the enzyme-inhibitor crystal structures. In particular, it appears that the absence of the covalent bond between Ser195

and **2** allows better hydrogen bonding and hydrophobic interactions to partially compensate for the binding energy provided by the Ser195 bond to **1**.

Acknowledgments

The authors would like to thank the following people for their contributions to this work: Ms. Jean F. Kaysen for preparation of the manuscript; Dr. Steve F. Brady and Mr. Ken J. Stauffer for the supply of intermediates; Dr. Jacquelynn J. Cook, Ms. Maria T. Stranieri, Ms. Marie A. Holahan, Dr. Jiunn H. Lin, Mr. I Wu Chen, and Ms. Kim B. Dancheck for pharmacokinetic studies; Drs. Paul S. Anderson, Paul A. Freidman, Joel R. Huff, Lawrence C. Kuo, and Jules A. Shafer for their support and encouragement; and the Analytical Chemistry Group for chemical characterization.

References

1. (a) Tapparelli, C.; Metternich, R.; Ehrhardt, C.; Cook, N. S. *Trends Pharmacol. Sci.* **1993**, *14*, 366. (b) Lyle, T. A. *Perspect. Drug Discovery Des.* **1993**, *1*, 453 and references sited therein. (c) Bode, W.; Mayer, I.; Baumann, U.; Huber, R.; Stone, R. S.; Hofsteenge, J. *EMBO J.* **1989**, *8*, 3417.
2. Brady, S. F.; Lewis, S. D.; Colton, C. D.; Stauffer, K. J.; Sisko, J. T.; Ng, A. S.; Homnick, C. F.; Bogusky, M. J.; Shafer, J. A.; Veber, D. F.; Nutt, R. F. *Proc. 14th Amer. Peptide Symp.* **1995**, 0.
3. (a) Brady, S. F.; Sisko, J. T.; Stauffer, K. J.; Colton, C. D.; Qiu, H.; Lewis, S. D.; Ng, A. S.; Shafer, J. A.; Bogusky, M. J.; Veber, D. F.; Nutt, R. F. *Bioorg. Med. Chem.* **1995**, *3*, 1063. (b) Lewis, S. D.; Ng, A. S.; Lyle, E. A.; Mellott, M. J.; Appleby, S. D.; Brady, S. F.; Stauffer, K. J.; Sisko, J. T.; Mao, S-S.; Veber, D. F.; Nutt, R. F.; Lynch, J. J.; Cook, J. J.; Gardell, S. J.; Shafer, J. A. *Thromb. Haemostasis* **1995**, *3*, 1063.
4. Cutrona, K. J.; Sanderson, P. E. J.; *Tetrahedron Lett.* **1996**, *37*, 5045.
5. K_i determinations were carried out as described in refs 3a,b and in: Lewis, S. D.; Ng, A. S.; Baldwin, J. J.; Fusetani, N.; Naylor, A. M.; Shafer, J. A. *Thromb. Res.* **1993**, *70*, 173.
6. Kurz, K. D.; Main, B. W.; Sandusky, G. E. *Thromb. Res.* **1990**, *60*, 269.
7. Lyle, E. A.; Lynch, Jr. J. J.; Cook, J. J.; Unpublished results.
8. Merck Research Laboratories Unpublished results.
9. Chen, Z.; Li, Y.; Mulichak, A. M.; Lewis, S. D.; Shafer, J. A. *Arch. Biochem. Biophys.* **1995**, *322*, 198.
10. Brunger, A. T.; Kuriyan, K.; Karplus, M. *Science* **1987**, *235*, 458.
11. Sack, J. S. *J. Mol. Graphics* **1988**, *6*, 224.
12. Coordinates have been deposited in the Brookhaven Protein Data Bank.

(*Received in USA* 21 *October* 1996; *accepted* 25 *November* 1996)