Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 1351–1356

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A NOVEL MOLECULAR DESIGN OF THROMBIN RECEPTOR ANTAGONIST

Tsugumi Fujita, Masahide Nakajima[1], Yoshihisa Inoue[1], Takeru Nose, and Yasuyuki Shimohigashi*

Laboratory of Structure-Function Biochemistry, Department of Molecular Chemistry, Graduate School of Science, Kyushu University, Fukuoka 812-8581, and [1]Research Division, The Green Cross Corp., Hirakata 573-1153, Japan

Received 22 January 1999; accepted 1 April 1999

Abstract: In a computer modeling of transmembrane domains of human thrombin receptor, Lys-158 was found near the ligand binding site. To capture this basic residue, analogs of peptide ligand containing a series of acidic amino acids were synthesized and assayed for human platelet aggregation, and Ser-(*p*-F)Phe-Aad(= α-aminoadipic acid)-Leu-Arg-Asn-Pro-NH_2 was found to be a potent antagonist.

The receptor of serine protease thrombin has a novel mechanism in activation. The receptor, a member of seven-transmembrane domain receptors, is cleaved by thrombin at the peptide bond between Arg-41 and Ser-42 in the *N*-terminal extension at the outer membrane, and a newly exposed *N*-terminal segment acts as a tethered ligand to activate the receptor itself.[1] A synthetic heptapeptide Ser-Phe-Leu-Leu-Arg-Asn-Pro-NH_2 (SFLLRNP in one letter amino acid codes) corresponding to this tethered ligand can activate the receptor without thrombin.[2,3] For better understanding of the molecular mechanism of receptor activation, antagonists are useful molecular tools to clarify the mode of the ligand-receptor interaction.

The antagonist for thrombin receptor would be also a remedy in the thrombosis therapeutics.[4] Several structural analogs of SFLLRNP have been announced as antagonist of the thrombin receptor, and in these cases modifications were focused mainly on the position 1; *i.e.*, substitution of Ser-1 by Tyr, deletion of Ser-1, and replacement of Ser-1 by β-mercaptopropionic acid.[5-8] However, none of these modifications elicited sufficient antagonist activities, and even contradictory results have been reported for the same compound in evaluation in the different bioassays.[5,9] In the molecular design of antagonist, it is often valid to introduce an additional structural element which interacts with a functional group in the receptor molecule.[10,11] If the interaction is very strong, a subsequent conformational change required for receptor activation would be disturbed and then the molecule would be an antagonist.

In the present study, in order to find such a functional group in the thrombin receptor, we carried out the computer modeling of protein molecule of thrombin receptor. When the lysine residues were searched, Lys-158 in the second transmembrane domain was found near the putative ligand binding site. Thus, we intended to capture this Lys by acidic amino acid loaded into SFLLRNP via the electrostatic interaction, and a series of SFLLRNP analogs were synthesized by substituting each amino acid residue with acidic amino acid Asp one after another.

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00202-4

Experimentals

Modeling of thrombin receptor. The three-dimensional structure of thrombin receptor was constructed on the basis of a multiple amino acid sequences alignment of seven-transmembrane domain receptors, after building a structure of β2 adrenergic receptor using the bacteriorhodopsin structure as template.[12] The modeling was performed using the software SYBYL 6.0.3 on an UNIX workstation model Indigo2 (Silicon Graphics Inc., Mt. View, CA, USA).

Peptide synthesis. Peptide syntheses were performed by the manual *t*-Boc or Fmoc strategy, using *p*-methylbenzhydrylamine resin or Fmoc-NH-SAL resin as reported previously.[13] Peptides liberated by HF or Reagent K (82.5% trifluoroacetic acid, 5% phenol, 5% water, 2.5% ethanedithiol, and 5% thioanisole) treatment were purified by gel filtration followed by preparative RP-HPLC (Hiber Pre-Packed column of 25 x 250 mm; average yield = 36%).

Platelet aggregation. Blood was obtained from healthy donors who denied taking any medications for the previous two weeks, and the assay was carried out using platelet-rich plasma as described.[14]

1. Search of receptor-functional groups near the ligand binding sites

Our previous results have suggested the existence of a highly specific aromatic π–π interaction between the Phe-2-phenyl group of SFLLRNP and the aromatic group of thrombin receptor.[13-15] Based on this π/π interaction, a binding site of SFLLRNP, the structure of which was optimized to an energy-minimized conformation, was searched on the computed molecular modeling of thrombin receptor.[12] When aromatic amino acid residues were highlighted, a cluster of several aromatic amino acids was found at the upper side of the fifth transmembrane domain. This cluster (—*YYAYYF*SA*F*SAV*FFF*—) (Phe (F) and Tyr (Y) in the italic letters) was assumed to be a binding site of Phe-2-phenyl. Taking this as a key interaction, a putative binding site of SFLLRNP was traced in the receptor molecule to find the best fitted structure that both the peptide and receptor join together in the outer space. The amino acid residues with functional side chain, for instance, acidic amino acids (Asp and Glu) or basic amino acids (Arg and Lys), were examined one after another, and then Lys-158 was found to be proximity to the ligand peptide. Lys-158 is present in the second transmembrane domain, which is in an α-helix structure (Fig. 1). As shown in Fig. 1, this basic amino acid residue situates its ω-aminobutyl side chain towards the inside of receptor molecule.

2. Design and syntheses of peptide ligands containing aspartic acid to capture receptor lysine-158

In order to capture Lys-158, Asp was incorporated into SFLLRNP at all the positions. Since a binding assay system for thrombin receptor has not been yet available because of the lack of a specific radio-ligand, synthetic peptides were directly assayed for their ability to aggregate the human platelet. The results are shown in Table 1. It should be noted that the analogs containing Asp at positions 1 ~ 5 exhibited no agonist activity. They were completely inactive. It is clear that Ser-1, Phe-2, Leu-3, Leu-4, and Arg-5 is crucially important in receptor activation and cannot compensate for the substitution with aspartic acid. Residual importance at these positions have been reported also by other studies.[16-18] In contrast, analogs Asp-6 and Asp-7 were totally active, eliciting a full platelet aggregation.

Since inactive peptides (Asp-1 ~ 5) were expected to have an antagonist activity, they were assayed for inhibition of platelet aggregation induced by the agonist. When peptides Asp-1, Asp-2, Asp-4, and Asp-5 (100 μM each) were added to the platelet rich plasma and incubated for 3 min, respectively, it was found that the

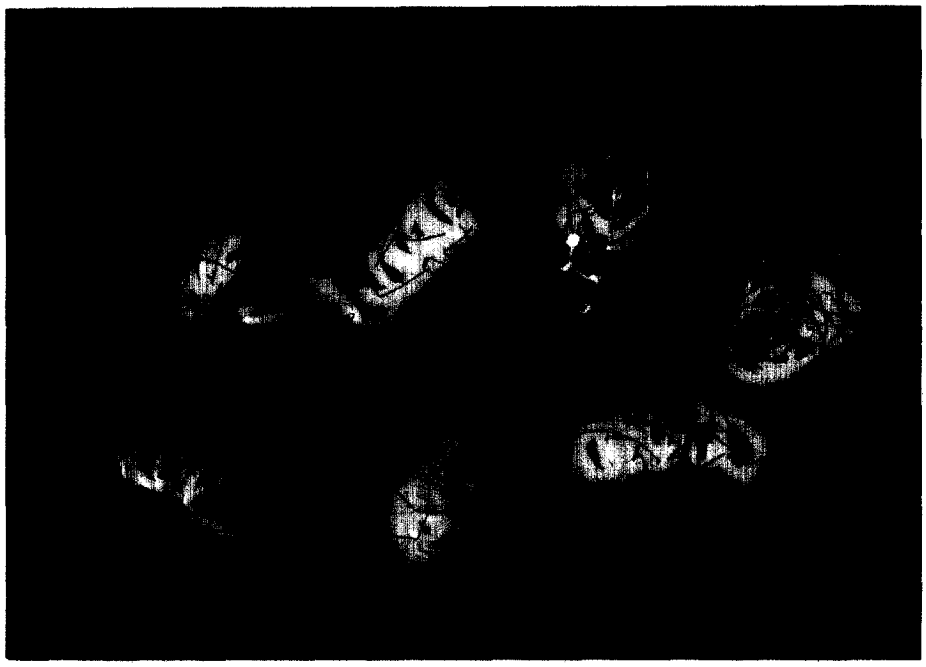

Fig. 1. The seven-transmembrane structure of thrombin receptor, in which Lys-158 is highlighted with balls and sticks (carbons in white, hydrogens in blue, and nitrogen in purple) in the second transmembrane domain. The Arabic numbers indicate the numerical order of transmembrane α-helix domains (yellow ribbons with amino acid side chains shown in red wireframes).

activity of SFLLRNP (1 μM) added afterwards has no influence at all. These analogs were completely devoid of both agonist and antagonist activities. However, when platelet-rich plasma was treated with SF/Asp/LRNP, the aggregation induced by SFLLRNP was clearly suppressed, not reaching to a full activation. This suppression by SF/Asp/LRNP was distinct (about 20 %) and reproducible. It was strongly suggested that the residue Asp-3 of SF/Asp/LRNP interacts with the receptor Lys-158 residue by the electrostatic interaction between Asp-β-COOH and Lys-ε-NH_2.

Table 1. Platelet aggregation activity of thrombin receptor tethered ligand peptide and its analogs.

Peptides		EC_{50} (μM)	Antagonist activity
SFLLRNP		4.27 ± 0.98	none
Asp/FLLRNP	Asp-1	inactive	none
S/Asp/LLRNP	Asp-2	inactive	none
SF/Asp/LRNP	**Asp-3**	**inactive**	**19%**
SFL/Asp/RNP	Asp-4	inactive	none
SFLL/Asp/NP	Asp-5	inactive	none
SFLLR/Asp/P	Asp-6	4.36 ± 0.88	none
SFLLRN/Asp	Asp-7	4.27 ± 0.88	none

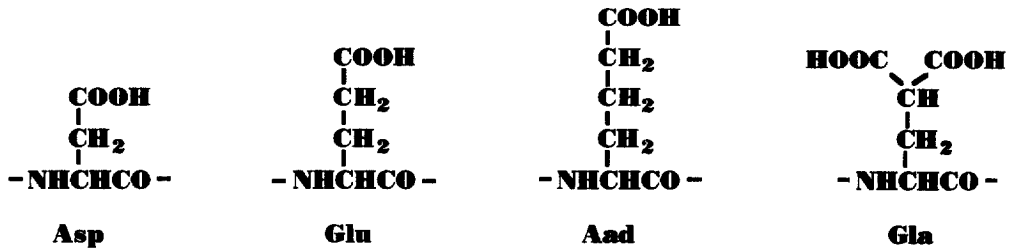

Fig. 2. The structure of side chain of acidic amino acids incorporated into a thrombin receptor-tethered ligand peptide SFLLRNP at the position 3.

3. Structure-activity relationships of peptide ligand containing a series of acidic amino acids

Since SF/Asp/LRNP exhibited only a limited antagonist activity, the interaction between Asp-β-COOH and Lys-ε-NH_2 appeared to be insufficient presumably owing to a shortage of the interacting distance. The side chain of Asp is the carboxymethyl group with a single methylene (Fig. 2). We further replaced this Asp-3 with L-glutamic acid and L-α-aminoadipic acid (Aad)[19] with two and three methylenes, respectively. Although both Glu-3 and Aad-3 derivatives were inactive to elicit a platelet aggregation, they showed a distinct antagonist activity. When the inhibitory activity of SFLLRNP derivatives with the acidic amino acids at position 3 were compared, it immediately became clear that the activity is dependent upon the number of methylenes. As shown in Fig. 3, SF/Glu/LRNP was more potent than SF/Asp/LRNP as an antagonist, exhibiting about 32% inhibition of platelet aggregation. SF/Aad/LRNP was more potent than these Asp and Glu derivatives with about 50% suppression. These results clearly indicate that the carboxyl group of Asp, Glu, and Aad is responsible for the antagonist activity of ligand peptide SF/Xaa (=acidic amino acids)/LRNP against the thrombin receptor, and thus the electrostatic interaction between these acidic residues and receptor amino group, presumably the one of Lys-158, appears to interfere the receptor activation.

γ-Carboxyglutamic acid (Gla) possesses two carboxyl groups on the γ-carbon of glutamic acid. When this Gla was placed at the position 3 instead of Glu of SF/Glu/LRNP, it was found that these Gla-3 and Glu-3 analogs exhibit almost the same inhibitory activity (32-34% suppression of human platelet aggregation at their 100 μM). This implies that the antagonist activity is contingent upon the distance of the carboxyl group from the peptide backbone, but not upon the number of carboxyl groups.

When Phe-2 of SFLLRNP was replaced by *para*-fluorophenylalanine ((*p*-F)Phe), the resulting S/(*p*-F)Phe/LLRNP exhibited considerably increased agonist activity (2-5-fold).[13-15] Thus, (*p*-F)Phe was introduced into a series of SF/Xaa/LRNP to examine whether or not (*p*-F)Phe is able to reinforce also the antagonist activity. It was found that the incorporation of (*p*-F)Phe increase approximately 20% the antagonist activity of parent SF/Xaa/LRNP (Fig. 3). In any pair of SF/Xaa/LRNP and S/(*p*-F)Phe/Xaa/LRNP, (*p*-F)Phe-2 derivatives were 20% more potent than Phe-2 derivatives. The most potent analog S/(*p*-F)Phe/Aad/LRNP exhibited a dose-dependent inhibition of platelet aggregation with the IC_{50} value of 115 μM. On the other hand, when the *N*-terminal Ser was removed from these antagonist peptides, the resulting hexapeptides (*p*-F)Phe/Xaa/LRNP showed considerably diminished (20%) antagonist activities, which were almost comparable to those of heptapeptide antagonists SF/Xaa/LRNP. Collectively, it is unequivocal that the peptide backbone of antagonist

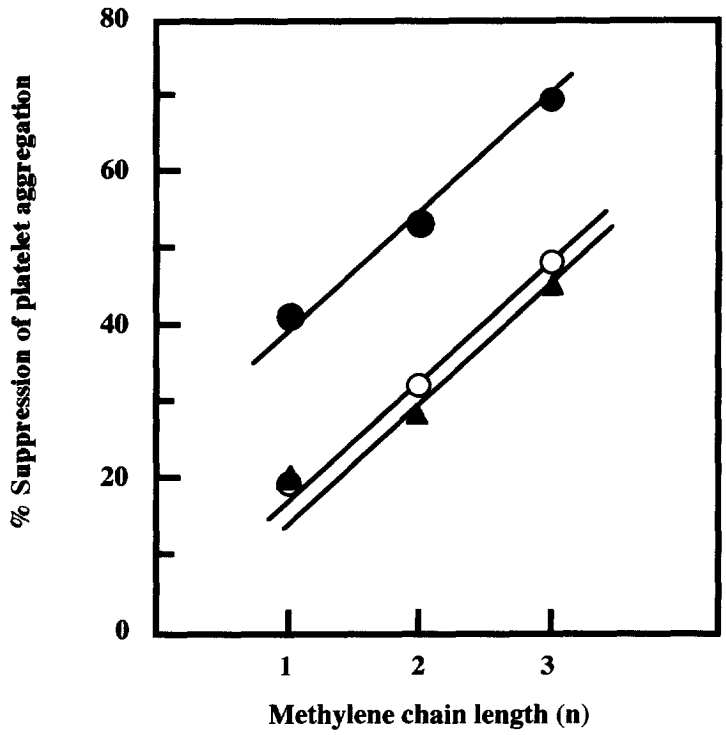

Fig. 3. The structure-activity relationship of heptapeptides SF/Xaa/LRNP in human platelet aggregation: Influence of side chain methylene length (n) of acidic amino acid residues Xaa, -NHCH($(CH_2)_n$COOH)CO-, at the position 3 on the suppression of aggregation. Xaa denotes the acidic amino acids Asp (n=1), Glu (n=2), and Aad (n=3). SF/X/LLRNP (○); S/(*p*-F)Phe/X/LRNP (●); and (*p*-F)Phe/X/LRNP (▲).

peptides is in an ordinary ligand-receptor interaction, while only the carboxyl group in the side chain at position 3 is sole structural element to bring about the antagonist activity.

It should be noted that SF/Asp/LRNP and its related peptides were inactive at their high concentrations, and inhibited a platelet aggregation induced by agonist SFLLRNP. This antagonist activity is certainly induced by the acidic amino acid at position 3 of antagonist SF/Xaa/LRNP. In this concern, the present results convince the idea that, for the molecular design of receptor antagonist, the peptide ligand should load an additional structural element which interacts with a functional group of the receptor molecule. The molecular design based on the receptor modeling is thus very favorable to obtain a pure antagonist. Since the potency of the present peptides to suppress the platelet aggregation was still very low, further receptor modeling would be helpful to find additional or alternative structural elements to improve the antagonist activity.

References and Notes:

1. Vu, T. -K. H.; Hung, D. T.; Wheaton, V. I.; Coughlin, S. R. *Cell* **1991**, *64*, 1057.
2. Rasmussen, U. B.; Vouret-Craviari, V.; Jallat, S.; Schlesinger, Y.; Pagès, G.; Pavirani, A.; Lecocq, J. -P.; Pouysségur, J.; Obberghen-Schilling, E. V. *FEBS Lett.* **1991**, *288*, 123-128.
3. Sakaguchi, K.; Kodama, H.; Ogino, Y.; Costa, T.; Nose, T.; Shimohigashi, Y. *Bull. Chem. Soc. Jpn.* **1994**, *67*, 1659.

4. Hung, D. T.; Vu, T. -K. H.; Wheaton, V. I.; Charo, I. F.; Nelken, N. A.; Esmon, N.; Esmon, C. T.; Coughlin, S. R. *J. Clin. Invest.* **1992**, *89*, 444.
5. Rasmussen, U. B.; Gachet, C.; Schlesinger, Y.; Hanau, Ohlmann P.; Obberghen-Schilling, E. V.; Pouyssegégur, J.; Cazenave, J.-P.; Paviran, A. *J. Biol. Chem.* **1993**, *268*, 14322.
6. Vassallo, R. R.; Kieber-Emmons, T.; Cichowski, K.; Brass, L. F. *J. Biol. Chem.* **1992**, *267*, 6081.
7. Tesfamariam, B. *Am. J. Physiol.* **1994**, *267*, H1962.
8. Seiler, S. T.; Peluso, M.; Michel, I. M.; Goldenberg, H.; Fenton, J. W.; Riexinger, D.; Natarajan, S. *Biochem. Pharmacol.* **1995**, *49*, 519.
9. Negrescu, E. V.; de Quintana, K. L.; Siess, W. *J. Biol. Chem.* **1995**, *270*, 1057.
10. Laufer, R.; Wormser, U.; Friedman, Z. Y.; Gilon, C.; Chorev, M.; Selinger, Z. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 7444.
11. Rivier, J. E.; Vale, W. W. *Life Sci.* **1978**, *23*, 869.
12. Henderson, R.; Baldwin, J. M.; Ceska, T. A.; Zemlin, F.; Beckmann, E.; Downing, K. H. *J. Mol. Biol.* **1990**, *213*, 899.
13. Nose, T.; Shimohigashi, Y.; Ohno, M.; Costa, T.; Shimizu, N.; Ogino, Y. *Biochem. Biophys. Res. Commun.* **1993**, *193*, 694.
14. Nose, T.; Fujita, T.; Nakajima, M.; Inoue, Y.; Costa, T.; Shimohigashi, Y. *J. Biochem.* **1998**, *124*, 354.
15. Shimohigashi, Y.; Nose, T.; Okazaki, M.; Satoh, Y.; Ohno, M.; Costa, T.; Shimizu, N.; Ogino, Y. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 366.
16. Nanevic, T.; Ishii, M.; Wang, L.; Chen, M.; Chen, J.; Turck, C. W.; Cohen, F. E.; Coughlin, S. R. *J. Biol. Chem.* **1995**, *270*, 21619.
17. Scarborough, R. M.; Naughton, M. A.; Teng, W.; Hung, D. T.; Rose, J.; Vu, T. -K. H.; Wheaton, V. I.; Turck, C. W.; Coughlin, S. R. *J. Biol. Chem.* **1992**, *267*, 13146.
18. Nose, T.; Shimohigashi, Y.; Okazaki, M.; Satoh, Y.; Costa, T.; Shimizu, N.; Ogino, Y.; Ohno, M. *Bull. Chem. Soc. Jpn.* **1995**, *68*, 2695.
19. Abbreviations: Aad, L-α-aminoadipic acid; Gla, L-γ-carboxyglutamic acid; SFLLRNP, amino acid sequence denoted by the one-letter amino acid code for Ser-Phe-Leu-Leu-Arg-Asn-Pro-NH_2 (All other peptides were also shown in a similar way); Xaa, acidic amino acids such as Asp, Glu, and Aad.