Development of Potent Thrombin Receptor Antagonist Peptides

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A peptide-based structure−activity study is reported leading to the discovery of novel potent thrombin receptor antagonists. Systematic substitution of nonproteogenic amino acids for the second and third residues of the human thrombin receptor "tethered ligand" sequence (SFLLR) led to a series of agonists with enhanced potency. The most potent pentapeptide agonist identified was Ser-*p*-fluoroPhe-*p*-guanidinoPhe-Leu-Arg-NH_2, **9** ($EC_{50} \sim 0.04\ \mu M$ for stimulation of human platelet aggregation, ∼10-fold more potent than the natural pentapeptide). Systematic substitution of the NH_2-terminal Ser in **9** with neutral hydrophobic NH_2-acyl groups led to partial agonists and eventually antagonists with unprecedented potency (greater than 1000-fold increase over the previously reported antagonist 3-mercaptopropionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH_2). In the series of NH_2-acyl tetrapeptide antagonists, *N-trans*-cinnamoyl-*p*-fluoroPhe-*p*-guanidinoPhe-Leu-Arg-NH_2, **41** (BMS-197525), was identified as the tightest binding ($IC_{50} \sim 8$ nM) and most potent with an $IC_{50} \sim 0.20\ \mu M$ for inhibition of SFLLRNP-NH_2-stimulated platelet aggregation. Systematic single substitutions in **41** indicated that, in addition to the NH_2-terminal acyl group, the side chains at the second and third positions were also responsible for important and specific receptor interactions. The *p*-fluoroPhe and *p*-guanidinoPhe residues in the second and third positions of **41** were observed to be optimal in both the agonist and antagonist series. In the case of antagonists, however, an appropriately positioned positively charged group (i.e., protonated base) at the third residue was required. In contrast, such a substitution was not required for potent agonist activity. An even more potent antagonist resulted when **41** was extended at the C-terminus by a single Arg residue giving rise to analog **90** (BMS-200261) which had an $IC_{50} \sim 20$ nM for inhibition of SFLLRNP-NH_2-stimulated platelet aggregation. When the C-terminal Arg of **90** was replaced by an Orn-(N^{δ}-propionyl) residue, the resulting antagonist **91** (BMS-200661) was suitable for use in radioligand binding assays ($K_d = 10-30$ nM). Antagonist activity observed for selected compounds was verified through secondary assays in that these analogs prevented SFLLRNP-NH_2-stimulated GTPase activity in platelet membranes and Ca^{2+} mobilization in cultured human smooth muscle cells and mouse fibroblasts. Furthermore, this inhibition occurred at concentrations that had no effect on thrombin catalytic activity indicating a specific activity attributable to receptor binding and not enzyme inhibition.

Introduction

Thrombin has been shown to have a variety of cellular actions that are mediated by proteolytic activation of a specific cell surface receptor known as the thrombin receptor (or "tethered ligand" receptor). The thrombin receptor is characterized by seven membrane-spanning domains[1] and is a member of the superfamily of G-protein-coupled receptors.[2] Activation of the receptor occurs by thrombin cleavage of an extracellular N-terminal domain thereby exposing a new NH_2-terminus which acts as a "tethered ligand" that intramolecularly binds to an appropriate site contained in the receptor structure.[1,3] By virtue of this structural rearrangement, the receptor becomes activated giving rise to various observable G-protein-coupled signal transduction pathways.[4]

The proteolytically activated thrombin receptor has been cloned[1,5−7] and shown to be present on numerous different cell types including human platelets,[1] endothelial cells,[6,8] fibroblasts,[5] vascular smooth muscle,[7,9] and cardiac myocytes.[10,11] A variety of responses are observable when such cells are treated with thrombin, and many of these actions can be mimicked by synthetic peptides corresponding to the N-terminal tethered ligand sequence. The identification of the thrombin receptor in relevant cell types, together with the realization that many of the pathophysiological actions of thrombin on these cells appear to be at least in part mediated by the thrombin receptor, suggests a role for this receptor in the pathological processes of thrombosis, inflammation, atherosclerosis, and fibroproliferative disorders.[1,5−11]

The development of thrombin receptor antagonists that may have value as therapeutic agents by specific inhibition of the cellular actions of thrombin (as opposed to its actions on clotting proteins) has been recognized,[4,12] but such an agent has not thus far been realized. Although limited peptide-based antagonists have been developed by analogy to the NH_2-terminal tethered ligand peptide of the thrombin receptor,[12−14] these compounds suffer serious limitations, specifically, lack of potency, partial agonist activity, and/or specificity.[4a]

An important objective of the work described in this report was to develop sufficiently potent, specific, and

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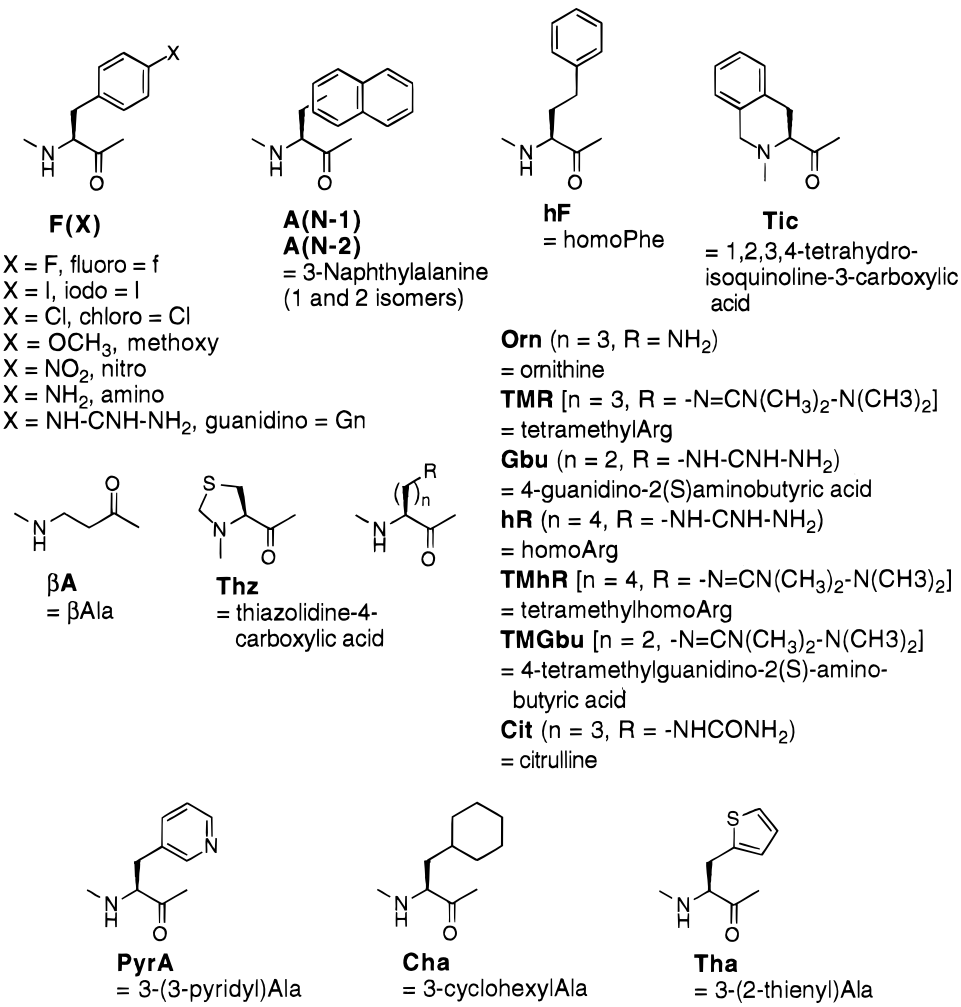

Figure 1. Structures and abbreviations used for nonproteogenic amino acid residues examined in the course of this study.

stable thrombin receptor antagonists that could be used for evaluation of their therapeutic potential.

In the abscence of precise structural information for the receptor, an "analog approach" was taken to antagonist development which involved cycles of systematic synthesis and testing. Previous studies of various thrombin receptor ligand peptides,[3,12,13,15−22] including a reported peptide-based structure−activity relationship (SAR) in which almost all of the natural amino acids were substituted, one at a time, for each residue of the natural biologically active "tethered ligand" sequence (SFLLR-NH_2, **2**), provided a basic understanding of the peptide ligand side chain structural requirements and their relative tolerances with regard to their interactions with the human thrombin receptor.[21] Although very few of these single amino acid substitutions produced a significant increase in agonist potency, an important finding was that substitution of the nonproteogenic 3-(2-naphthyl)alanine residue for leucine at position 3 of the natural sequence produced by far the most potent agonist (~4-fold > **2**) for human platelet aggregation found in that study.[21] A separate independent study indicated that substitution of *p*-fluorophenylalanine for phenylalanine at position 2 in SFLLRNP-NH_2 resulted in a 4−5-fold increase in activity as demonstrated by an assay measuring phosphoinositide turnover in human epithelial-like SH-EP cells.[20] The analog approach taken here was largely based upon additional substitution(s) with untested nonproteogenic amino acids.

This structure−activity excercise was expected to provide important information pertaining to (1) the elucidation of receptor binding mechanism(s), (2) the design and development of structural probes (e.g., photoaffinity labels), (3) the identification of peptides suitable for use to begin "computationally directed" screening of compound libraries for novel ligand types, (4) the development of novel antagonist-based binding assays (e.g., using an antagonist radioligand) for screening purposes, and (5) a basis for the design and development of novel nonpeptide antagonists. These objectives have at least been partially realized as a result of this study.

Results and Discussion

Agonist Optimization. Early on, the strategy employed here was to optimize agonist activity and then convert these to antagonists, if possible, by appropriate NH_2-terminal structural modification. Previous studies suggested that the use of nonproteogenic amino acids merited further exploration. Specifically, replacements with optimized unnatural residues, either singly or multiply in appropriate combination, might lead to further enhancements in binding and potency for known agonists. Furthermore analogs with nonproteogenic amino acid residues could have enhanced stability to proteases, an advantage if the peptides are to be studied in a biological setting. Figure 1 provides structures and

Table 1. Effect of Substitutions in the 2-Position of SFLLR-NH_2

peptide	mean EC_{50} (μM) (N)[a]
1, SF(f)LLR-NH_2	0.13 ± 0.05 (3)
2, SFLLR-NH_2 (human sequence)	0.40 ± 0.14 (3)
3, SF(OCH_3)LLR-NH_2	0.72 ± 0.15 (3)
4, SF(I)LLR-NH_2	9.27 ± 5.97 (3)
5, SF(NH_2)LLR-NH_2	18.00 ± 0.00 (3)
6, S-Tic-LLR-NH_2	110 ± 78.1 (3)
7, SF(Gn)LLR-NH_2	>300 (3)
8, S-hF-LLR-NH_2	>300 (3)

[a] Determined by platelet aggregation assay; EC_{50} = concentration required to stimulate 50% of maximum platelet aggregation, N = number of determinations. For amino acid abbreviations, see Figure 1.

Table 2. Effect of Substitutions at the 3-Position of Peptide **1**

peptide	mean EC_{50} (μM) (N)[a]
9, SF(f)F(Gn)LR-NH_2	0.04 ± 0.02 (3)
10, SF(f)F(f)LR-NH_2	0.10 ± 0.03 (3)
11, SF(f)A(N-2)LR-NH_2	0.08 ± 0.04 (3)
12, SF(f)A(N-1)LR-NH_2	0.16 ± 0.06 (3)
13, SF(f)-Tic-LR-NH_2	0.47 ± 0.19 (6)

[a] See Table 1.

abbreviations for the various nonproteogenic residues examined in the course of this study.

Table 1 summarizes the results of platelet aggregation assays on a series of peptide agonists in which the Phe residue at position 2 of the human sequence was replaced by various aromatic side chain-containing analogs. These data show that the binding pocket for the second side chain is exquisitely sensitive to size, conformational constraint, and electronic distribution. Of all the substitutions made in this series, only the *p*-fluoroPhe residue provided an enhancement in potency over the wild type human sequence **2**. The magnitude of the observed enhancement is in good agreement with previous reports for similar peptides.[20,22] It is clear from this and previously reported data[21] that the Phe binding site is very specific and that the *p*-fluoroPhe residue may represent a very nearly optimum substitution at position 2.

Table 2 summarizes the results of platelet aggregation assays on a series analogs with dual substitutions: the "optimized" *p*-fluoroPhe in position 2 and various residues at position 3. Substitution with both the *p*-fluoroPhe and 2-naphthylAla residues in peptide **11** produced an agonist with slightly enhanced potency (EC_{50} of 0.08 ± 0.04 μM) over that observed for either substitution alone (for reference, SFA(N-2)LR-NH_2 had an EC_{50} of 0.13 ± 0.07 μM).[21]

The *p*-guanidinoPhe replacement in the third position of peptide **9** was designed based on previous observations that a Phe substitution produced a peptide slightly greater in potency (~1.4-fold) than **2** and that an Arg substitution produced a somewhat greater (~1.7-fold) enhancement.[21] The *p*-guanidinoPhe residue was thus conceived as a hybrid combining potentially important structural aspects of the activity-enhancing residues: Phe, Arg, and 2-naphthylAla; this substitution in **9** produced the most potent peptide of this series with full agonist activity. As a result peptide **9** was utilized as a reasonable basis structure for further structural elaboration aimed at a crossover to antagonist activity, an approach that proved to be successful.

The results obtained for peptide **9** also suggested further investigation of minimum structural requirements for activity. The des-Arg tetrapeptide analog of **9** (SF(f)F(Gn)L-NH_2, **14**, BMS-194021) was synthesized and found to be an agonist with an EC_{50} of 0.28 ± 0.08 μM (N = 7). This is the most potent tetrapeptide agonist thus far published and more potent than the natural pentapeptide **2**. In contrast, the natural tetrapeptide SFLL-NH_2 was >400 times less potent than pentapeptide SFLLR-NH_2.[18] This shows that peptides with appropriate side chains as small as four residues can possess full activity and that five residues are not required as was previously thought.

It is also noteworthy that the conformational constraint imposed by a Tic residue in the third position of the peptide chain in analog **13** was relatively well tolerated, providing an analog nearly equal in potency to the natural sequence **2** but nonetheless substantially less potent than the other analogs of the series (Table 2).

Table 3. Effect of Preliminary N-Terminal Substitutions

peptide	mean EC_{50} (μM) (N)[a]
15, Ac-βAF(f)A(N-2)LR-NH_2	0.27 ± 0.03 (3)
16, Ac-βAFLLR-NH_2	1.80 ± 0.87 (3)
17, βAFLLR-NH_2	11.7 ± 4.51 (3)
18, Thz-FLLR-NH_2	46.0 ± 14.0 (3)
19, Ac-Thz-FLLR-NH_2	>300 (3)

[a] See Table 1.

Partial Agonist/Antagonist Discovery. Antagonists with modest potency had been reported which were based on the agonist structure where the Ser was replaced by a 3-mercaptopropionyl (Mpa) group[12,13] which suggested that antagonists might be achieved by substitution of Ser with neutral hydrophobic *N*-acyl groups. Initially derivatives of βAla and (*R*)-thiazolidine-4-carboxylic acid (Thz) were synthesized as near isosteric and conformationally constrained replacements for the Mpa group, respectively. The results are presented in Table 3. Very interestingly *N*-acetyl-βAla peptides **15** and **16**, although less potent than their Ser-containing counterparts, were found to be reasonably potent agonists. This result was somewhat surprising because, prior to this discovery, only peptides capable of providing a positively charged NH_2-terminus (i.e., a protonated amino group) were found to have appreciable agonist activity. Curiously, free amine peptide **17** was actually found to be less active in stimulation of platelet aggregation than its *N*-acetyl counterpart **16** (Table 3). This prompted additional biological evaluation including examination by GTPase assay.[21,23] Some peptides were capable of stimulating only a fraction of the total GTPase activity attainable by saturating levels of agonist peptide SFLLRNP-NH_2. For example, peptides **15**, **16**, and **18** were identified as partial agonists possessing 21%, 50−70%, and 33% intrinsic potency, respectively. This prompted further evaluation of NH_2-terminal substitutions in **9**. In the process, additional partial agonists and novel pure antagonists were identified. The antagonists were characterized by their ability to inhibit [^{3}H]SFFLRR-NH_2 binding to platelet membranes,[24] inability to stimulate platelet aggregation at concentrations >300 μM, and dose dependent inhibition of peptide agonist (or thrombin)-induced platelet aggregation, as well as their inability to stimulate the GTPase even at very high concentration. Tables 4 and 5 summarize these results.

Table 4. Effect of N-Terminal Variations in Early Generation Antagonist Peptides: X-F(f)F(Gn)LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
20	(2-thiophene)acetyl	0.26 (2)	32 ± 23 (4)
21	*N*-acetyl-2-aminobenzoyl	1.00 ± 0.31 (3)	ND
22	2-oxo(2-thiophene)acetyl	0.22 ± 0.08 (3)	47 ± 32 (4)
23	(3-thiophene)acetyl	0.31 ± 0.18 (3)	74 ± 38 (4)
24	phenylacetyl	0.68 ± 0.42 (3)	94 ± 37 (3)
25	(2-thiophene)sulfonyl	8.95 (2)	193 ± 23 (3)
26	(3-fluorophenyl)acetyl	0.16 ± 0.05 (3)	40 ± 19 (4)
27	(4-fluorophenyl)acetyl	0.35 ± 0.16 (3)	68 ± 17 (3)
28	3-pyridylacetyl	0.95 ± 0.34 (3)	>160 (4)
29	(2-fluorophenyl)acetyl	0.56 ± 0.14 (3)	35 ± 11 (4)
30	(3-indole)acetyl	0.50 ± 0.10 (3)	54 ± 8 (4)
31	cyclopentylacetyl	0.37 ± 0.17 (3)	85 ± 26 (4)
32	2-oxo(3-indole)acetyl	0.13 ± 0.08 (3)	9.9 ± 6.5 (3)
33	3-indoloyl	0.019 ± 0.011 (3)	2.0 ± 0.7 (3)
34	(3-chlorophenyl)acetyl	0.11 (2)	2.9 ± 1.1 (3)

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] IC_{50} = concentration required for 50% inhibition of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation.

Table 5. Effect of N-Terminal Structure (X) on Peptides (X-F(f)F(Gn)LR-NH_2) Identified as Partial Agonists

peptide	X	IC_{50} (μM)[a] (*N*)	EC_{50} (μM)[b] (*N*)
35	*N*-acetyl-4-aminobutyryl	0.27 ± 0.16 (3)	8.7 (3)
36	2-thiopheneoyl	0.025 ± 0.014 (3)	0.10 ± 0.07 (4)
37	3-thiopheneoyl	0.024 ± 0.008 (3)	PA[c]
38	3-furanoyl	0.029 ± 0.004 (3)	PA[c]
39	2-indoloyl	0.024 ± 0.003 (3)	1.8 (2)
40	4-chlorobenzoyl	0.026 (2)	3.7 (2)

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] EC_{50} = concentration required for stimulation of 50% maximum platelet aggregation. [c] PA = partial agonists for which maximum platelet aggregation was not experimentally achieved.

In the course of generating and evaluating the data (Tables 4 and 5), simple generalizations became possible. Whenever a relatively small aryl ring system was directly attached to the carbonyl group of the NH_2-terminal amide, partial agonists resulted. Inclusion of a spacer group such as a methylene or additional carbonyl group between the aryl ring and the amide carbonyl resulted in antagonists. From this data it was hypothesized that antagonists that provided a structure capable of extending greater distances from the NH_2-terminal amide, possibly with some conformational constraint, could result in enhanced binding by virtue of additional favorable interactions with the receptor that are unavailable to shorter NH_2-termini. To test this possibility an NH_2-terminal *trans*-cinnamoyl group, containing a conformationally restrictive and chain-extending olefinic linker between an aryl ring and the N-terminal amide group, was incorporated into an analog in this series (peptide **41**). Upon its biological evaluation, peptide **41** (Table 6) was found to be the tightest binding and most potent NH_2-acyl tetrapeptide antagonist thus far discovered. The antagonist activity of compound **41** was confirmed through additional activity assays (for more details, see Antagonist Validation section). Table 6 summarizes data obtained for **41** and related NH_2-acyl tetrapeptide analogs, many of which were also found to be reasonably potent antagonists.

Antagonist Development. Despite the relatively large number of NH_2-acyl group replacements for the *trans*-cinnamoyl moiety in **41** (Tables 4−6), only conservative modifications of the NH_2-terminal cinnamoyl group of **41** were well tolerated. NH_2-Terminal structures giving rise to antagonists with binding assay IC_{50} values <50 nM are given in Figure 2. These results indicate a high degree of structural specificity for the receptor binding site interacting with the NH_2-termini of these analogs and an important functional role for this interaction dictating the type of activity observed for a particular analog (agonist vs partial agonist vs antagonist). The data also suggest that the phenyl ring in the cinnamoyl group in **41** binds with a preferred conformation (twisted out of the plane of the adjacent olefin), as more rigid but reasonably conservative analogs **53** and **54** demonstrate dramatic reductions in binding and potency.

Another specific receptor interaction important for both binding and antagonist function was recognized by substituting the *p*-guanidinoPhe residue of **41** with various aromatic, neutral, or basic residues (Tables 7−9). Here again, the lead analog **41** remained the tightest binding and most potent antagonist. These data indicate a requirement for a positively charged group (protonated amine or guanidine) in order to obtain tight binding antagonist activity. Replacement of the guanidino group of **41** with hydrogen (analog **69**) produced a very significant reduction in binding and, remarkably, a crossover to some agonist activity. Appropriate positioning of the charge with respect to the rest of peptide as well as the size of the charged group are also important factors influencing binding and function. For example, Lys, homoArg, and *N*,*N*′-tetramethylhomoArg relacements (analogs **79**−**81**) gave rise to partial agonist activity, while replacement with Arg (**76**) was better tolerated resulting in an antagonist with slightly reduced affinity and potency. These data, taken together with the SAR obtained for NH_2-terminal variants, suggest that the arylguanidine of **41** plays an important role in orienting or positioning the the NH_2-terminal cinnamoyl group for an optimum antagonist generating interaction with the receptor.

Effect of Peptide Length/Antagonist Radioligand Development. The effects of C-terminal deletions and additions or replacements in anlogs of lead peptide **41** were also examined (Tables 10 and 11). Very interestingly, binding at the sub-micromolar level was retained in a molecule as small as the NH_2-acyl dipeptide analog **83**, and the potency of this dipeptide was comparable to that of a number of NH_2-acyl tetrapeptide antagonists (Table 4) having less optimal NH_2-termini. Such results suggest that it may be possible to prepare relatively small molecule peptidomimetic thrombin receptor antagonists with useful potency. When the CO_2H-terminal Arg was deleted from **41** to give analog **84**, significant reduction in both binding and potency are observed but reasonable antagonist activity is retained. The contribution of the CO_2H-terminal Arg of **41** to binding and potency is nonetheless substantial.

SAR reported for peptide agonists[3,15−18,22] suggested that the binding and potency of **41** may be enhanced by the inclusion of an additional CO_2H-terminal residue; furthermore, if an Orn residue could be tolerated at this position, acylation of its side chain amine with a radiolabled acylating agent might provide a useful radioligand for development of antagonist-based binding assays. The results obtained for anlogs with CO_2H-terminal replacements or extensions are given in Tables

Table 6. Effect of N-Terminal Structure (X) on *N*-Acyl Tetrapeptide Series: X-F(f)F(Gn)LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
41	*trans*-cinnamoyl	0.0078 ± 0.0033 (3)	0.20 ± 0.07 (4)
42	3-phenyl-2-propynoyl	0.023 ± 0.014 (3)	0.28 ± 0.20 (6)
43	*p*-fluoro-*trans*-cinnamoyl	0.020 ± 0.003 (3)	0.09 ± 0.04 (3)
44	*p*-chloro-*trans*-cinnamoyl	0.099 ± 0.006 (3)	0.24 ± 0.06 (6)
45	*p*-methyl-*trans*-cinnamoyl	0.034 ± 0.008 (3)	0.95 ± 0.83 (5)
46	*p*-methoxy-*trans*-cinnamoyl	0.040 ± 0.023 (3)	0.39 ± 0.13 (3)
47	4-biphenyloyl	0.046 ± 0.014 (3)	0.43 ± 0.25 (5)[c]
48	*m*-chloro-*trans*-cinnamoyl	0.041 ± 0.020 (3)	0.08 ± 0.04 (4)[d]
49	3-phenylpropionyl	0.042 ± 0.007 (3)	1.19 ± 0.56 (5)
50	phenoxyacetyl	0.021 ± 0.003 (3)	0.26 ± 0.08 (4)
51	1-naphthylacetyl	1.63 ± 0.67 (3)	3.87 ± 1.94 (3)
52	3-(2-thiophene)-*trans*-acryloyl	0.023 ± 0.003 (3)	0.88 ± 0.44 (4)
53	(±)-*trans*-3-phenylcyclopropanoyl	2.32 ± 0.93 (3)	3.00 ± 1.31 (3)
54	3-coumarinoyl	4.33 ± 0.15 (3)	21.7 ± 14.5 (3)
55	4-phenylbutyryl	1.26 ± 1.00 (3)	3.93 ± 1.68 (3)
56	*p*-amino-*trans*-cinnamoyl	0.061 ± 0.013 (3)	1.63 ± 0.25 (3)
57	2-naphthylacetyl	0.295 ± 0.057 (3)	4.67 ± 1.92 (3)[e]
58	*p*-hydroxy-*trans*-cinnamoyl	0.062 ± 0.015 (3)	2.73 ± 0.58 (3)
59	(thiophenoxy)acetyl	0.019 ± 0.007 (3)	0.69 ± 0.16 (3)
60	*trans*-2-*trans*-4-hexadienoyl	0.024 ± 0.009 (3)	PA[f]
61	*trans*-2-octenoyl	0.035 ± 0.013 (3)	7.60 ± 9.02 (3)
62	α-fluorocinnamoyl	0.023 ± 0.006 (3)	0.53 ± 0.27 (3)
63	α-methylcinnamoyl	0.043 ± 0.001 (3)	8.33 ± 6.81 (3)
64	α-phenylcinnamoyl	1.53 ± 0.15 (3)	66.7 ± 11.6 (3)

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] IC_{50} = concentration required for 50% inhibition of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation. [c–e] These peptides displayed well-resolved mixed pharmacology with agonist (platelet aggregation) activity EC_{50}'s of [c]18 μM (N = 2), [d]120 μM (N = 2), and [e]90 μM (N = 1). [f] PA = partial agonist for which maximum platelet aggregation was not experimentally achieved.

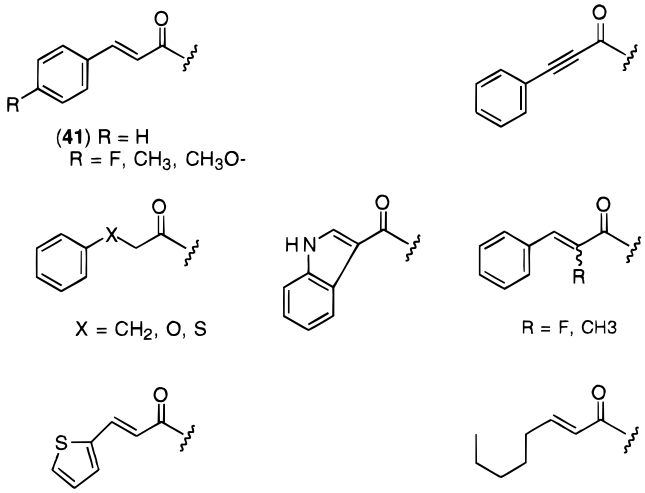

Figure 2. Best tolerated (IC_{50} < 50 nM, binding) N-terminal structures in antagonist analogs of **41**.

Table 7. Effects of Hydrophobic Aromatic Replacements (X) for the *p*-GuanidinoPhe Residue in Analogs of **41**: *trans*-Cinnamoyl-F(f)-X-LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
65	homoPhe	0.63 ± 0.026 (3)	>300 (2)
66	*p*-nitroPhe	7.55 ± 10.4 (4)	>300 (2)
67	*p*-chloroPhe	4.71 ± 1.0 (3)	>300 (2)
68	*p*-methoxyPhe	1.18 ± 0.13 (3)	>300 (2)
69	Phe	1.12 ± 0.33 (4)	17 (2)[c]
70	β-(2-naphthyl)Ala	0.246 ± 0.018 (3)	5 (1)[c]

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] IC_{50} = concentration required for 50% inhibition of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation. [c] These peptides displayed agonist activity with the specified EC_{50} values.

11 and 12. Addition of a basic residue (Orn or Arg in analogs **88** and **90**, respectively) resulted in analogs with marginally improved binding but a more significant positive impact on antagonist potency (about a 5−10-fold enhancement over **41**) was observed. When the Orn residue of **88** was acetylated (**89**) or propionylated (**91**), a slight reduction in binding (<3-fold) was observed, but these analogs were still more potent at inhibiting platelet aggregation than **41**. Given these results, peptide **91** was targeted for radioligand synthesis. After propionylation of **88** in solution with *N*-succinimidyl-[2,3-^{3}H]propionate (Amersham), HPLC-purified tritiated **91** was obtained with a specific activity of 80 Ci/mmol and a radiochemical purity of >98% (P. Egli, unpublished results). Using this tritiated **91**, an antagonist-

Table 8. Effects of Hydrophophilic Neutral or Weakly Basic Aromatic Replacements (X) for the *p*-GuanidinoPhe Residue in Analogs of **41**: *trans*-Cinnamoyl-F(f)-X-LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	EC_{50} (μM)[b] (*N*)
71	citrulline	1.04 ± 0.06 (4)	67 (2)
72	His	0.47 ± 0.11 (3)	35 ± 22 (4)[c]
73	β-(3-pyridyl)Ala	0.34 ± 0.02 (3)	116 ± 100 (4)[c]
74	*p*-aminoPhe	0.37 ± 0.03 (3)	60

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] EC_{50} = concentration required for stimulation of 50% maximum platelet aggregation. [c] Concentration required for 50% inhibition (i.e., IC_{50}) of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation.

Table 9. Effects of Aliphatic Basic (i.e., Positively Charged) Residue Replacements (X) for the *p*-GuanidinoPhe Residue in Analogs of **41**: *trans*-Cinnamoyl-F(f)-X-LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
75	Orn	0.17 ± 0.08 (3)	8.70 ± 3.63 (4)
76	Arg	0.028 ± 0.016 (3)	1.13 ± 0.67 (3)
77	TMR	0.32 ± 0.13 (3)	16 (2)
78	Gbu	0.028 ± 0.007 (3)	8.0 (1)
79	Lys	0.039 ± 0.002 (3)	18 (1)[c]
80	hR	0.012 ± 0.002 (3)	0.5 (1)[c]
81	TMhR	0.13 ± 0.03 (3)	90 (1)[c]
82	TMGbu	0.34 ± 0.18 (3)	140 (1)

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] IC_{50} = concentration required for 50% inhibition of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation. [c] These peptides displayed agonist (or partial agonist) activity with the specified EC_{50} values.

Table 10. Effects of C-Terminal Truncation in Analogs of **41**: *trans*-Cinnamoyl-F(f)-F(Gn)-X

peptide	X	IC_{50} (μM)[a] (N)	IC_{50} (μM)[b] (N)
83	NH_2	0.78 ± 0.20 (3)	64 (2)
84	Leu-NH_2	0.11 ± 0.09 (3)	4.05 ± 2.66 (4)
85	Orn-NH_2	0.87 ± 0.13 (3)	141 (2)

[a,b] See Table 9.

Table 11. Effects of C-Terminal Replacements and Additions to Analogs of **41**: *trans*-Cinnamoyl-F(f)-F(Gn)L-X

peptide	X	IC_{50} (μM)[a] (N)	IC_{50} (μM)[b] (N)
86	Orn-NH_2	0.017 ± 0.003 (3)	0.19 ± 0.09 (3)
87	Orn(acetyl)-NH_2	0.044 ± 0.002 (3)	0.25 ± 0.22 (3)
88	Arg-Orn-NH_2	0.0066 ± 0.002 (3)	0.047 ± 0.018 (4)
89	Arg-Orn(acetyl)-NH_2	0.0166 ± 0.006 (3)	0.040 ± 0.020 (4)
90	Arg-Arg-NH_2	0.0075 ± 0.001 (3)	0.021 ± 0.004 (3)
91	Arg-Orn(pro)[c]-NH_2	0.0219 ± 0.004 (3)	0.085 ± 0.025 (4)

[a,b] See Table 9. [c] pro = propionyl.

based binding assay was developed (S. M. Seiler, unpublished results) showing competitive binding with various agonists and antagonists, as well as distinctly different kinetics of binding compared to the normal agonist radioligand. Those studies will be detailed separately.

Results of replacing the NH_2-terminal *trans*-cinnamoyl group in the most potent analog **90** with some of the best tolerated replacements identified previously in the NH_2-acyl tetrapeptide series (Table 6, Figure 2) are given in Table 12. In this case, the NH_2-terminal 3-phenyl-2-propynoyl group was found to provide the tightest binding ($IC_{50} \sim 4$ nM) antagonist **94** (BMS-201516) thus far discovered, but it was comparable in platelet aggregation inhibition potency to its *trans*-cinnamoyl counterpart **90**. Thus, in addition to a positively charged residue (e.g., *p*-guanidinoPhe) at position 3 possibly having some role in positioning the critical NH_2-terminus at the receptor binding site, such NH_2-terminal positioning may also be influenced by interactions sequentially more remote (i.e., at the more CO_2H-terminal side chains of positions 5 and 6). Overall the results reported here for CO_2H-terminally extended antagonists are consistent with and parallel those observed for peptide agonists for which optimal potency was observed at the length of hexapeptides.[22]

Effect of 2-Position Substitutions in Antagonists. Because the *p*-fluorophenyl side chain represented an optimal substitution at the 2-position in the peptide agonists, this specific and important interaction was further probed in the tight binding NH_2-acyl tetrapeptide antagonist series by various systematic substitutions. The results are presented in Table 13. As observed for agonist peptides, the *p*-fluorophenyl side chain provides an optimal and apparently specific and important interaction. Conservative substitution with a phenyl side chain in analog **97** was tolerated but resulted in significant losses in binding (~3-fold) and potency (>8-fold). Furthermore a single replacement of the *p*-fluorophenyl side chain of **41** by a hydrogen (Gly analog **96**) produced a peptide that was almost devoid of activity. Clearly a marked preference for a hydrophobic aromatic residue has been demonstrated at this position.

Antagonist Validation. Because of the unprecedented binding and antagonist activity observed for peptide **41**, additional biological characterization was carried out. Peptide **41** blocked platelet aggregation stimulated by 3 μM SFLLRNP-NH_2 with an IC_{50} of 0.2 ± 0.07 μM ($N = 4$) and caused concentration dependent rightward shifts in the concentration of agonist required for platelet aggregation (Figure 3), indicating that the compound is a competitive antagonist for SFLLRNP-NH_2 activation of platelets. The inhibition of platelet aggregation gave a Schild slope of 1.02 and a pA_2 of 7.26 ($N = 2$). This inhibition was specific to the thrombin receptor in that platelet aggregation stimulated by ADP and U-46619, a thromboxane receptor agonist, was unaffected. Peptide **41** also inhibited thrombin-induced platelet aggregation, but its potency against SFLLRNP-NH_2 was greater than against thrombin. The IC_{50} for inhibition of thrombin-induced platelet aggregation also depended on the thrombin concentration and the individual platelet donor. This result is consistent with enzymatic cleavage of the receptor generating a tethered ligand having a very high effective local concentration, thereby reducing the likelihood of inhibition by a competitive antagonist at less than saturating concentrations.

Antagonist **41** also inhibited SFLLRNP-NH_2 and thrombin-stimulated membrane GTPase activity in platelet and CHRF-288 cell membranes. The inhibition of SFLLRNP-NH_2-stimulated platelet membrane GTPase is shown in Figure 4. In human aortic smooth muscle cells (HASMs), most of the intracellular Ca^{2+} mobilization observed with SFLLRNP-NH_2 was prevented by **41** (Figure 5). As for the platelet aggregation studies, a higher concentration of **41** was required to inhibit HASM activation by thrombin compared to SFLLRNP-NH_2. Analog **41** also inhibited SFLLRNP-NH_2-stimulated Ca^{2+} mobilization in mouse Swiss 3T3 cells (data not shown). These and other studies indicate that the antagonists reported here inhibit the thrombin receptor in at least several different species and tissues.

Furthermore **41** was found not to inhibit thrombin proteolytic activity[25] ($K_i = 52$ μM for inhibition of thrombin by **41**) at levels where antagonist responses were observed showing that its biological activity was thrombin receptor specific.

Summary

Beginning with existing SAR obtained for thrombin receptor agonist peptides and preparing novel analogs by systematically substituting residues with nonproteogenic amino acids, first singly and then in combination, it was possible to derive the most potent agonist pentapeptide, SF(f)F(Gn)LR-NH_2 (**9**), reported to date. Single substitution of the NH_2-terminal Ser in agonist **9** with a wide variety of neutral hydrophobic NH_2-acyl groups led to the identification and discovery of novel partial agonists and antagonists. The most potent NH_2-acyl tetrapeptide antagonist derived from **9** was *trans*-cinnamoyl-F(f)F(Gn)LR-NH_2 (**41**) which exhibited specific binding to the human thrombin receptor at unprecedented single-digit nanomolar levels (>1000-fold enhancement over reported peptide antagonists) and inhibited agonist-induced platelet aggregation with an IC_{50} potency of ~0.2 μM. Data obtained for NH_2-terminal acyl group substitution analogs of **41** suggest a specific and important interaction for the *trans*-cinnamoyl moiety of **41**. Further SAR by systematic substitutions of the *p*-fluoroPhe and *p*-guanidinoPhe residues of **41** did not lead to improved binding or antagonist potency indicating important and specific

Table 12. Effects of Single (X) or Dual (X, Z) Replacement in Analogs of **90**: X-F(f)-F(Gn)-LR-Z-NH_2

peptide	X	Z	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
92	*trans*-4-fluorocinnamoyl	Arg	0.019 ± 0.007 (3)	0.05 ± 0.01 (3)
93	3-phenylpropionyl	Arg	0.009 ± 0.001 (3)	0.25 ± 0.12 (3)
94	3-phenyl-2-propynoyl	Arg	0.004 ± 0.001 (3)	0.040 ± 0.019 (3)
95	*trans*-4-fluorocinnamoyl	Orn(acetyl)	0.025 ± 0.005 (3)	0.060 ± 0.010 (3)

[a,b] See Table 9.

Table 13. Effects of Substitutions (X) for *p*-FluoroPhe in Analogs of **41**: *trans*-Cinnamoyl-X-F(Gn)LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
96	Gly	≫30	>100 (2)
97	Phe	0.024 ± 0.007 (4)	1.73 ± 0.46 (3)
98	Cha[c]	0.093 ± 0.032 (4)	8.73 ± 4.95 (3)
99	Tha[c]	0.042 ± 0.011 (4)	3.31 ± 0.57 (3)
100	PyrA[c]	3.44 ± 1.14 (4)	>55 (3)

[a,b] See Table 9. [c] See Figure 1 for structures and abbreviations.

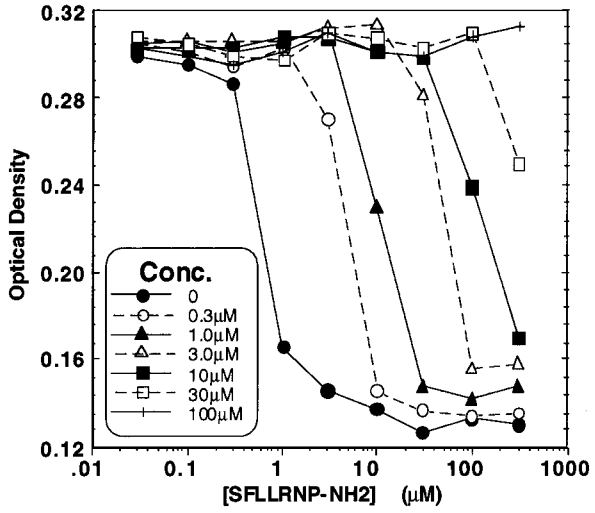

Figure 3. Inhibition of SFLLRNP-NH_2-stimulated human platelet aggregation by peptide **41**. Platelet aggregation was measured using gel-filtered human platelets in a microtiter turbidity assay as previously described.[21,35] The platelets were preincubated with the indicated concentrations of **41** prior to stimulation by SFLLRNP-NH_2. This experiment is an example of several yielding similar results.

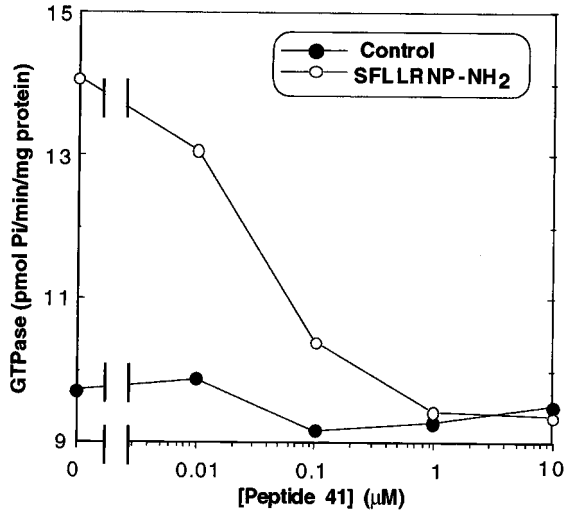

Figure 4. Inhibition of SFLLRNP-NH_2-stimulated platelet membrane GTPase activity by peptide **41**. Platelet membrane GTPase was measured with (○) and without (●) 20 μM SFLLRNP-NH_2. Thrombin receptor-stimulated GTPase was determined as described previously.[23] This experiment is an example of three yielding similar results.

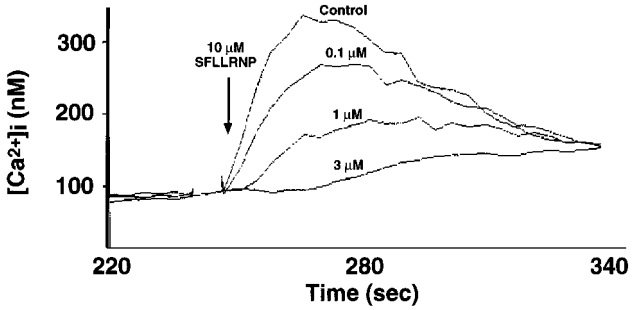

Figure 5. Inhibition of SFLLRNP-NH_2-stimulated Ca^{2+} mobilization in cultured human aortic smooth muscle (HASM) cells. The cells were loaded with the fura-2 Ca^{2+} fluorescent indicator by incubation with fura-2 AM. The loaded cells were washed and preincubated with the indicated concentrations of peptide **41**. Ca^{2+} mobilization was induced by addition of 10 μM SFLLRNP-NH_2 (arrow). Fluorescence (excitation at 340 and 380 nm with emission at 505 nm) was measured.

interactions with the receptor at those peptide sites in addition to those identified at the NH_2-terminus. Also noteworthy is an apparent requirement for an appropriately positioned positively charged group (i.e., protonatable base) to mimic the guanidinophenyl side chain of **41** if tight binding and potent antagonism are to be realized. In general the antagonist SAR thus obtained parallels that obtained for agonists. These observations, combined with the ability of peptide agonists and antagonists to compete for receptor binding, suggest at least partially common modes of binding for both types with distinct and functional differences residing mainly at the NH_2-termini that dictate what type of activity (GTPase coupling or uncoupling, i.e., agonist or antagonist) is observed for a particular structure. Furthermore it appears that the critical NH_2-terminal interaction is at least in part influenced by interactions downstream with more CO_2H-terminal side chains. Thus, extension of **41** by inclusion of an additional Arg residue at the CO_2H-terminus gave the most potent antagonist **90** with similar receptor binding affinity to **41** but with a significant increase (~10-fold, IC_{50} ~ 20 nM) in potency. When the NH_2-terminal *trans*-cinnamoyl group of **90** was replaced with a 3-phenyl-2-propynoyl group, the tightest binding antagonist **94** (IC_{50} ~ 4 nM) resulted, which had potency comparable to **90**. Given the specificity, unprecedented binding affinity, and potency for the thrombin receptor antagonists disclosed here, it is likely that these, or specifically designed derivatives of these, will serve as valuable tools for elucidation of thrombin receptor structure, function, and pharmacology and serve as a starting point for new drug discovery. Indeed, a practically accessible tritiated antagonist (**91**) has been shown to be a useful radioligand suitable for use in binding assays.

Experimental Section

Materials. Boc-Leu-OH, Boc-Arg(Tos)-OH, and *p*-methylbenzhydrylamino-polystyrene resins (1% DVB cross-linked,

0.56 and 0.36 mequiv/g) were purchased from Peninsula Laboratories (Belmont, CA). All nonproteogenic Boc-amino acids were from Bachem Bioscience (Philadelphia, PA) except Boc-Phe(*p*-*N*,*N*′-bis-Cbz-guanidine)-OH which was prepared by reaction of Boc-Phe(*p*-NH_2)-OH with *N*,*N*′-bis-Cbz-1-guanylpyrazole[26] as described previously.[27] Trifluoroacetic acid was from Eastman Kodak (Rochester, NY), diisopropylethylamine was from Fluka (Buchs, Switzerland), and BOP and HBTU reagents were from Midwest Biotech (Fishers, IN). Coupling reagent solution, 1 M DCC in NMP, was from Applied Biosystems (Foster City, CA). Carboxylic acids used for N-terminal acylations, 2-thiophenesulfonyl chloride, and anhydrous anisole were from Aldrich (Milwaukee, WI). Synthesis solvents (CH_2Cl_2, DMF, diethyl ether) were obtained from Fisher Scientific (Pittsburgh, PA). Hydrogen fluoride was from Matheson (East Rutherford, NJ). All commercial chemicals used were of the highest quality available (AR grade or better).

Peptide Synthesis. All peptides were prepared manually using standard solid phase synthesis techniques and the Boc/benzyl protection strategy. Syntheses were performed starting from 0.08−0.10 mmol of *p*-methylbenzhydrylamino-polystyrene resin. Boc group removals were carried out by treatment with TFA/CH_2Cl_2 (1:1) for 15 min. Neutralization was performed by two brief washes with 5% DIEA in CH_2Cl_2. Couplings were performed using 4 equiv of Boc-amino acid (or 3 equiv for nonproteogenic Boc-amino acids) with equivalent amounts of BOP reagent[28] and DIEA in minimum DMF and were monitored for completion using the Kaiser ninhydrin test.[29] NH_2-terminal acylations by carboxylic acids in general were conducted similarly (i.e., BOP/DIEA) except that 10 equiv were used. In some cases, where BOP-derived intermediates precipitated during preactivation, carboxylic acids were coupled using 1 M DCC in NMP. In the case of the NH_2-terminal phenylpropioloyl group (analogs **42**, **94**), N-acylation was conducted using the *N*-hydroxysuccinimide ester of phenylpropiolic acid (prepared by reaction of phenylpropiolic acid with DCC and *N*-hydroxysuccinimide in THF at 4 °C, 2 h, then at room temperature, 2 h) in CH_2Cl_2/NMP. In some cases, arginine residues were introduced starting with Boc-Orn(Fmoc)-OH, and after usual peptide chain assembly followed by specific cleavage of the side chain Fmoc protection (10% diethylamine in DMF, 10 min) from the protected peptidyl-resin, the guanidino group was incorporated using 1*H*-pyrazole-1-carboxamidine hydrochloride in DMF/DIEA[30] in the last solid phase chemistry step before final HF deprotection and cleavage. Peptides containing *N*,*N*′-tetramethylguanidine side chains were prepared similarly from the appropriate N^{α}-Boc-N^{ω}-Fmoc-amino acid using HBTU/DIEA (10 equiv each) in minimal DMF (3 h, room temperature) to incorporate the tetramethylguanidino group.[31] Peptides with NH_2-acetyl and NH_2-propionyl side chains were also prepared by this strategy (i.e., via orthogonal Fmoc protection of side chain amines which, after deprotection, were acylated using standard reagents while still resin-bound). Completed protected peptidyl-resins were cleaved and deprotected using HF containing 5% anisole at 4 °C for 1 h. After HF removal *in vacuo*, the products were washed several times with diethyl ether and extracted with several portions into 20−30 mL of 5% HOAc in water. The entire solution of crude product thus obtained was purified by loading directly onto a preparative HPLC system with a C-18 column using a linear gradient of increasing acetonitrile in water containing 0.1% TFA for elution as previously detailed.[32] Fractions shown by HPLC to be >95% pure were pooled and lyophilized to provide, with a few exceptions, 25−45 mg of peptide products (ca. 30−50% overall yield) as white powder TFA salts that in general were >98% pure as determined by HPLC (YMC C_{18} column, detection at 215 and 280 nm using several gradients and solvent systems).

All peptides were found to have, within experimental variation (±5%), the expected composition as determined after hydrolysis[33] and amino acid analysis using the Pico Tag method,[34] which was also used to determine solution concentrations. Peptide identity was further confirmed by FAB mass spectral analysis with molecular ion peaks at $(M + H)^+$ and $(M - H)^-$ in negative ion mode, observed for all peptides. The ^{1}H NMR data for lead antagonist tetrapeptide **41** is representative: The spectrum (600 MHz) was obtained on a 3 mM solution of **41** in D_2O (pH 6.6) at 3 °C, and peak positions in δ (ppm) (peak positions for exchangeable amide NH's were determined with H_2O as solvent) are reported relative to residual HDO in solvent which resonates 5.02 ppm downfield from TSP. NMR: δ 0.89 (s, 3H, Leu CH_3), 0.955 (s, 3H, Leu CH_3), 1.48 (m, 1H, Leu γ CH), 1.635 (m, 3H, Leu γ CH, Leu β CH_2), 1.675 (m, 2H, Arg γ CH_2), 1.785 (m, 1H, Arg βCH_2), 1.88 (m, 1H, Arg βCH_2), 2.915 (m, 1H, Phe(Gn) βCH_2), 3.02 (m, 2H, Phe(fluoro) βCH_2), 3.13 (m, 1H, Phe(Gn) βCH_2), 3.20 (m, 2H, Arg δ CH_2), 4.25 (m, 1H, Arg α CH), 4.29 (m, 1H, Leu α CH), 4.59 (m, 1H, Phe(Gn) α CH), 4.63 (m, 1H, Phe(fluoro) α CH), 6.57 (d, 1H, J = 15.3 Hz, cinnamoyl α vinyl), 6.68 (d, 1H, C-terminal NH), 6.96 (d, 1H, C-terminal NH), 7.05 (m, 2H, aryl), 7.095 (m, 2H, aryl), 7.21 (m, 2H, aryl), 7.245 (m, 2H, aryl), 7.27 (m, 1H, Arg ϵ NH), 7.45 (d, 1H, cinnamoyl β vinyl overlapped with 7.49, m, 3H, cinnamoyl aryls), 7.66 (m, 2H, aryl), 8.22 (d, 1H, J = 6.8 Hz, Leu NH), 8.33 (d, 1H, J = 7.8 Hz, Phe(Gn) α NH), 8.42 (d, 1H, J = 7.0 Hz, Arg α NH), 8.50 (d, 1H, J = 7.6 Hz, Phe(fluoro) NH).

Biological Assays. Assays for human platelet aggregation activity,[21,35] platelet membrane GTPase activity,[21,23] and receptor binding assays using 20−25 nM [^{3}H] SFFLRR-NH_2 and a platelet membrane preparation were performed as described elsewhere.[24]

Abbreviations. Abbreviations for amino acids and nomenclature of peptide structures not specifically stated in the text follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1971**, *247*, 997). Other abbreviations not given in the text are as follows: Boc = *tert*-butyloxycarbonyl; BOP = (1*H*-benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate; Cbz = benzyloxycarbonyl; DCC = *N*,*N*′-dicyclohexylcarbodiimide; DIEA = diisopropylethylamine; DMF = dimethylformamide; DVB = divinylbenzene; FAB = fast atom bombardment; Fmoc = [(9-fluorenylmethyl)oxy]carbonyl, HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMP = *N*-methylpyrrolidinone; SAR = structure−activity relationship; Tos = tosyl (*p*-toluenesulfonyl); TSP = (trimethylsilyl)[2,2,3,3-$^2H_4$]propionate.

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