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Discovery and Structure–Activity Relationship Studies of a Novel and Specific Peptide Motif, Pro-X-X-X-Asp-X, as a Platelet Fibrinogen Receptor Antagonist

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Abstract—A novel hexapeptide, H-Pro-Ser-Nva-Gly-Asp-Trp-OH **6**, a specific antagonist of platelet fibrinogen receptor (GpIIb/IIIa), was discovered in a structure–activity relationship (SAR) study where the role of the N-terminal Pro moiety of an RGD-containing peptide, H-Pro-Ser-Arg-Gly-Asp-Trp-OH **1**, which is a potent but not specific antagonist toward GpIIb/IIIa integrin, was investigated. This novel peptide **6** exhibits very high activity as a human platelet aggregation inhibitor (IC_{50} 0.59 μM, human PRP/collagen) as well as marked specificity for GpIIb/IIIa. A series of substitutions at the third position (Nva residue) in this hexapeptide, focused on the conformational rigidity, led to compounds which are superior to the original novel peptide **6** with regard to anti-platelet activity. The peptides, H-Pro-Ser-Hyp-Gly-Asp-Trp-OH **17** and H-Pro-Ser-ΔPro-Gly-Asp-Trp-OH **18** with the 5-membered ring structure, which restricted the conformation of the peptide backbone at the third position, inhibited the aggregation of human platelets at submicromolar concentrations (IC_{50} 0.39 and 0.30 μM, respectively). Further structure–activity relationship studies at each position of the peptide sequence suggest a novel motif sequence, Pro-X1-X2-X3-Asp-X4, for specific GpIIb/IIIa integrin recognition, in which the N-terminal free Pro residue and the Asp residue at the fifth position are essential to the activity. This motif sequence is summarized as follows: (1) a small amino acid such as Ser, Ala or Gly is preferable at X1 position; (2) X2 may be any amino acid, preferably a bulky amino acid such as Tle or a cyclic amino acid such as Pro; (3) X3 must be a small amino acid such as Gly; and (4) X4 is preferably an amino acid with an aromatic side chain.

Introduction

Platelet aggregation and thrombosis play an important role in various vasoocclusive diseases such as unstable angina, myocardial infarctions, transient ischemic attacks and stroke.[1–3] In addition, during extracorporeal circulation, undesired platelet aggregation, induced by the interaction between the platelet and fibrinogen-coated artificial surface of the extracorporeal circuit, is suspected to be the major cause of profound platelet loss and platelet dysfunction leading to bleeding.[4,5] Platelet aggregation is generally mediated by fibrinogen which can specifically bind to the platelet membrane glycoprotein IIb/IIIa (GpIIb/IIIa) on activated platelets, and this step is the critical event leading to platelet aggregation in thrombus formation.[6,7] Therefore, inhibition of this GPIIb/IIIa-mediated event constitutes an attractive antithrombotic mechanism.

GpIIb/IIIa is a member of integrins which are heterodimeric cell surface receptor molecules and are thought to be particularly important mediators of cell adhesion, cell migration and adhesion-dependent intracellular signalling. Many of the integrins recognize Arg-Gly-Asp (RGD) sequence as a common recognition motif within

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their putative ligands upon ligand–receptor interaction.[7–13] Synthetic studies showed the potential of peptides containing the RGD sequence to inhibit the binding of fibrinogen to GpIIb/IIIa and, thereby, to inhibit platelet aggregation and thrombus formation.[14–16] Thereafter, many small peptides containing the RGD sequence have been synthesized and their activity and specificity towards platelet GpIIb/IIIa were evaluated to develop anti-platelet aggregatory agents.[17] Most of the linear RGD-containing peptides have shown very low specificity among many types of integrin receptors including platelet GpIIb/IIIa, αvβ3 (vitronectin receptor) and α5β1 (fibronectin receptor). However, it has also been reported that some RGD peptides with a cyclic structure show relatively high specificity.[18–20] These results suggest that the three-dimensional structure of RGD site itself and/or its flanking regions are very important for the ligand-binding specificity because cyclic peptides have very rigid structure and their three-dimensional structures are considerably fixed. This idea is also supported by the fact that RGD-containing snake venom peptides, disintegrins,[21] such as echistatin, kistrin or mambin, have shown very low specificity toward integrins.[22] The reason for this low specificity can be explained by the fact that the RGD domain of these peptides is on an exposed, flexible loop.[23–25]

On the other hand, several peptides without the RGD sequence, which are specific for platelet GpIIb/IIIa, have also been reported. For example, a tick saliva protein, disagregin,[26] which does not have an RGD sequence, is a potent GpIIb/IIIa antagonist and shows high specificity for this integrin. Other examples are barbourin,[27] a member of the disintegrins found in snake venom, and a decapeptide, LGGAKQAGDV, from the carboxyl terminus of the fibrinogen γ-chain.[28,29]

Because peptides are easily digested into non-toxic inactive forms in vivo, it seems that peptide GpIIb/IIIa antagonists prevent prolonged bleeding time which is one of the major side effects of anti-platelet agents, while a number of non-peptide antagonists of GpIIb/IIIa with high specificity have recently been reported.[17,30]

The aim of our study is to develop a potent and highly specific GpIIb/IIIa antagonist of small linear oligopeptides for anti-thrombotic agents. Recently, we have described the biological character of a novel and specific hexapeptide motif, Pro-X-X-X-Asp-X, for GpIIb/IIIa recognition.[31] In this report, we describe the discovery of this novel peptide motif from one of our potent RGD-containing peptides, Pro-Ser-Arg-Gly-Asp-Trp **1** (IC_{50} 0.87 μM, platelet aggregation), described in a previous study,[32] and the structure–activity relationship (SAR) study of this novel peptide motif for anti-platelet aggregation activity. It has been thought that the arginine residue in the RGD-containing peptide is one of the essential amino acids for integrin recognition. These new motif peptides with no arginine residue, which exhibit potent binding activity and high specificity for GpIIb/IIIa, are provided as a useful tool in integrin research as well as agents in anti-thrombotic therapy.

Results

All the peptides were synthesized by solid-phase methods,[33] purified by RP-HPLC, and characterized by mass spectrometry and amino acid analysis. The purity of the synthetic peptides was assessed by TLC (single spot by ninhydrin detection in three different solvent systems) and HPLC (single peak by UV detection at 225 nm) (Table 9).

Discovery of a novel peptide sequence for fibrinogen receptor recognition

As previously reported,[32] an RGD-containing hexapeptide, Pro-Ser-Arg-Gly-Asp-Trp, peptide **1** (Table 1), was a very potent inhibitor of platelet aggregation (IC_{50} 0.87 μM). The modification of the N-terminal Pro residue

Table 1. Modifications of PSRGDW

No.	Peptide[a]	Platelet aggregation[b] IC_{50}(μM)	GpIIb/IIIa ELISA IC_{50}(μM)
1	PS-**Arg**-GDW	0.87	0.051
2	N-Ac-PS-**Arg**-GDW	50	1.8
3	N-Me-PS-**Arg**-GDW	20	N.d.
4	Thf-S-**Arg**-GDW	21	N.d.
5	homoPro-S-**Arg**-GDW	11	N.d.
6	PS-**Nva**-GDW (NSL-9507)	0.59	0.030
7	PS-**Ala**-GDW	4.7	0.22
8	PS-**Val**-GDW	7.0	N.d.
9	PS-**Leu**-GDW	1.3	0.10
10	PS-**Nle**-GDW	0.87	0.025
11	PS-**Ile**-GDW	1.1	0.024
12	PS-**alloIle**-GDW	1.1	0.033
13	PS-**Tle**-GDW	0.65	0.027
14	PS-**Chg**-GDW	0.75	0.019
15	PS-**Phg**-GDW	0.75	0.026
	GRGDS	470	13
	SRGDW	40	N.d.

[a]N-Ac; N-acetyl, N-Me; N-methyl, Thf; (±)-tetrahydro-2-furancarbonyl, homoPro; L-pipecolinic acid, Nva; L-norvaline, Nle; L-norleucine, allolle; L-allo-isoleucine, Tle; L-tert-leucine, Chg; L-cyclohexylglycine, Phg; L-phenylglycine.
[b]Collagen (5 μg/ml)-induced platelet aggregation using human platelet-rich plasma.
N.d., Not determined.

such as N-acetylation (peptide **2**), N-methylation (peptide **3**) or the replacement of the nitrogen atom with an oxygen atom (peptide **4**), decreased the inhibitory activity by 20 to 60 times compared to that of peptide **1**. Moreover, the change in the ring size (the nitrogen heterocycle) from 5 to 6 in the Pro residue of peptide **1** decreased its activity (peptide **5**). These results indicated that the free secondary amino (imino) moiety of this N-terminal Pro residue is significant for potent activity, suggesting that the strict spatial arrangement of the imino group gives a new site for an ionic interaction with GpIIb/IIIa. However, this peptide was not specific for platelet GpIIb/IIIa integrin, although it was very potent. In general, the presence of both the guanidino group (basic moiety) of the Arg residue and the β-carboxylic acid (acidic moiety) of the Asp residue in RGD-containing peptides is essential to the recognition by many integrin receptors as a common recognition motif. Therefore, in order to acquire linear peptides with high specificity toward one integrin such as GpIIb/IIIa, it was presumed that the RGD structure in the integrin-recognizing peptides should be partially changed and especially the above-mentioned two moieties that associate with integrins were the targets worth changing. The exceptionally potent activity of peptide **1** led us to the idea of elimination of the guanidino group from the Arg residue to obtain high specificity toward GpIIb/IIIa (Figure 1). We synthesized peptide **6**, Pro-Ser-Nva-Gly-Asp-Trp, NSL-9507, in which the Arg residue in peptide **1** has been replaced with a norvaline (Nva) residue, and its anti-platelet activity was evaluated. As shown in Table 1, peptide **6** showed a slight increase in both the anti-platelet activity (IC_{50} 0.59 μM) and the inhibitory activity of the fibrinogen binding to GpIIb/IIIa receptor (IC_{50} 0.030 μM). This result suggests that the Arg residue of the RGD motif is not necessary for the anti-platelet activity in this peptide.

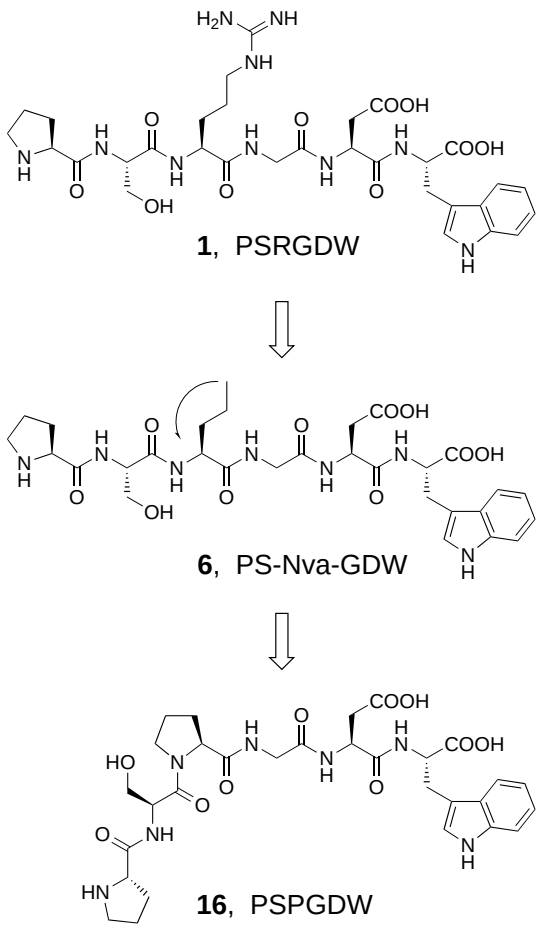

Figure 1. Discovery of a novel motif sequence for fibrinogen receptor antagonists.

SAR study of Nva position in peptide 6 (NSL-9507)

Encouraged by the discovery of a novel hexapeptide **6**, with potent anti-platelet activity but without an RGD motif, we have synthesized a series of peptides, whose Nva residue is substituted with neutral amino acids having different types of side chains, to elucidate factors that affect the anti-platelet activity. The activity of these peptides is shown in Table 1. Although the replacement of the norvaline residue with relatively small amino acids such as alanine (peptide **7**) or valine (peptide **8**) resulted in a decrease in the anti-platelet activity, replacement with bulky amino acids such as *tert*-leucine (Tle) (peptide **13**) and cyclohexylglycine (Chg) (peptide **14**) or relatively large amino acids such as Nle (peptide **10**) and Ile (peptide **11**) retained the same activity as that of norvaline, suggesting that amino acids with steric hindrance are preferable at this position for potent activity, with the exception of leucine (peptide **9**) which showed slightly low activity.

In order to reduce the flexibility of the peptide and enhance the conformational rigidity at this position, we introduced amino acids with a cyclic structure to the third residue (Figure 1). When L-proline, which has a 5-membered ring structure, was introduced, anti-platelet activity at a submicromolar concentration was obtained (peptide **16**, Table 2). The effects of the introduction of some proline derivatives were also examined. As shown in Table 2, peptide **17** (NSL-9511) that has a hydroxyproline (Hyp) residue at the third position and peptide **18** that has a 3,4-dehydroproline residue (ΔPro) both inhibited platelet aggregation with an IC_{50} of 0.39 and 0.30 μM, respectively. Peptide **18** was the most potent one out of more than 100 peptides we synthesized. The introduction of a pyrrolidine ring that has a large or bulky side chain such as 4-benzyloxy group (peptide **21**) and 3,3-dimethyl group (peptide **20**) did not greatly

affect the anti-platelet activity, indicating that the space around the third residue is not so restricted. However, the replacement of L-proline with D-proline resulted in the loss of activity, suggesting that L-form amino acids, not D-form, are required. The change in the ring size from 5 to 4 (peptide **23**) or to 6 (peptide **24**) also decreased the activity.

H-Pro-Xl-X2-X3-Asp-X4 as the novel motif peptide for anti-platelet activity

As shown in Table 3, the modification of the imino group of the N-terminal Pro residue (peptide **25**) resulted in complete loss of the activity, although the peptides with the same modification of this group in peptide **1** showed the anti-platelet activity at micromolar concentrations (Table 1), suggesting that the N-terminal Pro residue with a free imino group is essential to the peptide without the Arg residue. The Asp residue at the fifth position was also essential because the substitution of this residue for a Glu residue (peptide **26**) or an Ala residue (peptide **27**) resulted in almost complete loss of the activity. These results lead us to a common motif sequence, Pro-X1-X2-X3-Asp-X4, in which X1 to X4 are L-form α-amino acids.

As shown in Table 4, the peptides, to which a small amino acid (such as alanine **28** or glycine **29**) or a basic amino acid (such as arginine **33** or histidine **34**) was introduced at the X1 position, showed anti-platelet activity that was 25 to 50 times weaker than that of peptide **16** (Ser residue at this position). The introduction of a bulky (Ile, Phe, or Tyr) or an acidic amino acid (Glu) did not reproduce the potent activity.

The effects of the substitution at the X2 residue with some different types of L-form natural α-amino acids were also studied. As shown in Table 5, the influence of the substitutions was relatively small. By contrast, only the Gly residue was acceptable for the anti-platelet

Table 2. Modifications of PS-Nva-GDW

No.	Peptide[a]	Platelet aggregation[b] $IC_{50}(\mu M)$	GpIIb/IIIa ELISA $IC_{50}(\mu M)$
16	PS-**Pro**-GDW (NSL-9510)	0.77	0.017
17	PS-**Hyp**-GDW (NSL-9511)	0.39	0.018
18	PS-**ΔPro**-GDW	0.30	0.018
19	PS-**Thz**-GDW	0.51	0.019
20	PS-**Dmt**-GDW	0.65	0.019
21	PS-**Hyp(Bzl)**-GDW	0.55	0.013
22	PS-D-**Pro**-GDW	340	210
23	PS-**Azt**-GDW	5.1	0.27
24	PS-**homoPro**-GDW	3.7	0.20

[a]Hyp; L-4-hydroxyproline, ΔPro; 3,4-dehydro-L-proline, Thz; L-thiazolidine-4-carboxylic acid, Dmt; L-dimethylthiazolidine-4-carboxylic acid, Hyp(Bzl); O-benzyl-L-hydroxyproline, Azt; L-2-azetidinecarboxylic acid, homoPro; L-pipecolinic acid.
[b]See Table 1.

Table 3. Modifications of **PSPGDW**

No.	Peptide[a]	Platelet aggregation[b] $IC_{50}(\mu M)$	GpIIb/IIIa ELISA $IC_{50}(\mu M)$
25	**N-Ac**-PSPGDW	> 1000	> 1000
26	PSPG-**Glu-W**	> 1000	> 1000
27	PSPG-**Ala-W**	> 1000	80

[a]N-Ac; N-acetyl.
[b]see Table 1.

Table 4. Modifications of PSPGDW at the second (X1) residue

No.	Peptide P-(**X1**)-PGDW	Platelet aggregation[a] $IC_{50}(\mu M)$	GpIIb/IIIa ELISA $IC_{50}(\mu M)$
16	P-**Ser**-PGDW NSL-9510	0.77	0.017
28	P-**Ala**-PGDW	19	0.40
29	P-**Gly**-PGDW	36	0.65
30	P-**Ile**-PGDW	> 1000	9.0
31	P-**Met**-PGDW	410	45
32	P-**Phe**-PGDW	730	14
33	P-**Arg**-PGDW	36	7.2
34	P-**His**-PGDW	36	4.3
35	P-**Tyr**-PGDW	> 1000	650
36	P-**Glu**-PGDW	760	120

[a]See Table 1.

Table 5. Modifications of PSPGDW at the third (X2) residue

No.	Peptide PS-(**X2**)-GDW	Platelet aggregation[a] $IC_{50}(\mu M)$	GpIIb/IIIa ELISA $IC_{50}(\mu M)$
16	PS-**Pro**-GDW (NSL-9510)	0.77	0.017
37	PS-**Ser**-GDW	4.9	0.11
38	PS-**Met**-GDW	1.6	0.17
39	PS-**Phe**-GDW	1.4	0.18
40	PS-**Lys**-GDW	1.4	0.058
41	PS-**His**-GDW	3.1	0.068
42	PS-**Tyr**-GDW	3.1	N.d.
43	PS-**Glu**-GDW	5.5	0.072

[a]See Table 1.
N.d., Not determined.

activity at submicromolar concentrations at the X3 position, because the peptides with an amino acid other than glycine at this position showed extremely low activity.

At the X4 position, aromatic amino acids were relatively preferable as compared with other amino acids (Table 7). The effects of the modification of the C-terminal carboxylic group were also examined. The amidation of this group resulted in a slight decrease in the antithrombotic activity (compare the IC_{50} values of peptide **16** and peptide **64**). Peptide **65**, which had an additional Arg residue at the C-terminal, inhibited platelet aggregation at submicromolar concentrations.

Table 6. Modifications of PSPGDW at the fourth (X3) residue

No.	Peptide PSP-(**X3**)-DW	Platelet aggregation[a] IC_{50}(μM)	GpIIb/IIIa ELISA IC_{50}(μM)
16	PSP-**Gly**-DW (NSL-9510)	0.77	0.017
44	PSP-**Ala**-DW	84	0.22
45	PSP-**Ser**-DW	400	3.3
46	PSP-**Thr**-DW	> 1000	20
47	PSP-**Pro**-DW	94	N.d.
48	PSP-**Val**-DW	> 1000	30
49	PSP-**Met**-DW	1200	N.d.
50	PSP-**Ile**-DW	> 1000	N.d.
51	PSP-**Phe**-DW	> 1000	N.d.
52	PSP-**Arg**-DW	50	0.80
53	PSP-**His**-DW	1100	14
54	PSP-**Tyr**-DW	> 1000	N.d.
55	PSP-**Glu**-DW	640	N.d.

[a]See Table 1.
N.d.; Not determined.

Table 7. Modifications of PSPGDW at the sixth (X4) residue

No.	Peptide PSPGD-(**X4**)	Platelet aggregation[a] IC_{50}(μM)	GpIIb/IIIa ELISA IC_{50}(μM)
16	PSPGD-**Trp** (NSL-9510)	0.77	0.017
56	PSPGD-**Gly**	24	N.d.
57	PSPGD-**Ser**	4.1	0.061
58	PSPGD-**Ile**	5.1	0.10
59	PSPGD-**Met**	6.4	0.12
60	PSPGD-**Phe**	1.4	0.027
61	PSPGD-**Arg**	5.1	0.074
62	PSPGD-**Tyr**	1.6	0.028
63	PSPGD-**Glu**	43	N.d.
64	PSPGD-**Trp-NH_2**	3.1	0.13
65	PSPGD-**Trp-Arg**	0.60	N.d.

[a]See Table 1.
N.d.; Not determined.

Integrin specificity of motif peptide

To clarify the specificity of the newly discovered peptide motif toward integrins, we have examined the effects of synthesized peptides on the fibrinogen binding to GpIIb/IIIa and the vitronectin binding to αvβ3 using receptor binding assay.[19] As Table 8 shows, the novel motif peptides with the inhibitory activity of the fibrinogen binding to GpIIb/IIIa did not inhibit the vitronectin binding to αvβ3, indicating that they are completely specific for GpIIb/IIIa.

Discussion

We have been developing fibrinogen receptor (GpIIb/IIIa) antagonists for anti-thrombotic agents, consisting of both peptide and non-peptide compounds[34] based on the RGD sequence which is one of the recognition sites on fibrinogen for platelet integrin GpIIb/IIIa. In the present study, we have found a series of potent and highly specific antagonists of GpIIb/IIIa which are linear hexapeptides without the RGD sequence represented as a sequence of Pro-X1-X2-X3-Asp-X4. Although several peptide antagonists with high specificity for this integrin have already been reported, such as a C-terminal decapeptide of the fibrinogen γ-chain,[28,29] a tick saliva protein,[26] barbourin[27] or synthetic cyclic peptides,[18–20] this is the first report of small linear peptides consisting of L-form natural amino acids.

The advantages of the present peptides as an antithrombosis agent are that (1) they are potent and very specific antagonists of GpIIb/IIIa in spite of a flexible linear structure, (2) they are easily synthesized by solid phase methods, and (3) some of them are peptides consisting of the natural amino acids, which are quickly digested to form non-toxic compounds (amino acids) in vivo. This may have potency to prevent the increasing

Table 8. Integrin selectivity of the synthetic peptides

No.	Peptide[a]	αvβ3 ELISA IC_{50}(μM)
6	PSNvaGDW	> 100
16	PSPGDW	> 100
17	PSHypGDW	> 100
28	PAPGDW	> 100
34	PHPGDW	> 100
44	PSPADW	> 100
52	PSPRDW	> 100
58	PSPGDI	> 100
64	PSPGDW-NH_2	> 100
65	PSPGDWR	> 100
	GRGDS	2.0

[a]Nva; L-norvaline, Hyp; L-4-hydroxyproline.

tendency of bleeding which occurs due to the administration of long acting and strong anti-thrombotic agents.[35–37]

The peptides developed here demonstrated that the N-terminal free Pro residue (basic moiety) and the β-carboxylic acid of the Asp residue (acidic moiety) at the fifth position in the sequence was critical for anti-platelet activity, suggesting that these two functional groups are the major interaction sites with platelet GpIIb/IIIa, because the modification of the imino group of the N-terminal Pro residue and substitution of the Asp residue with the other amino acid result in complete loss of the activity.

Moreover, the N-terminal Pro residue was important to provide specific binding to GpIIb/IIIa. The secondary amino group as a basic moiety for the specific GpIIb/IIIa antagonists has already been reported in several studies regarding to the non-peptide antagonists.[30,38] Although these secondary amino groups are mostly a 4-piperidyl group (6-membered ring), the 2-pyrrolidinyl group (5-membered ring) was preferable for potent anti-platelet activity in the present peptides, because peptide **5** which has a 4-piperidine ring structure at the N-terminal showed weak activity. These observations suggest the importance of spatially proper location of the N-terminal imino group. However, it is difficult to determine that both imino groups recognize the same interaction site on the GpIIb/IIIa receptor. Additionally, from the results in which the novel motif peptides did not inhibit the vitronectin binding to αvβ3, it is suggested that the secondary amino group is not eligible as a basic moiety for αvβ3 recognition, although GpIIb/IIIa can accept both guanidino and imino groups.

Upon substitution of the Nva residue at the third position of peptide **6** by either an amino acid with a bulky side chain or a Pro residue, a potent anti-platelet activity was maintained, and further modification of the pyrrolidine ring of this Pro residue can slightly enhance the anti-platelet activity. However, replacement with a D-Pro or a change in ring size (a nitrogen heterocyde) decreased the anti-platelet activity. From these results, we can assume that the conformational restriction of the peptide backbone at the third position by the pyrrolidine ring is more closely related to the active conformation for anti-platelet activity. This assumption is supported by the result that a more conformation-restricted analogue of the pyrrolidine ring, 3,4-dehydroproline (peptide **18**) showed the highest inhibitory activity among these analogues. The great activity obtained by the substitution with bulky amino acids at this position such as peptide **13** and **14** can also be explained by the same effect in that the conformational bending of the peptide backbone may be caused by bulky side chains with large steric hindrance such as a pyrrolidine structure. However, it is difficult to determine whether the effect of the *n*-propyl group of an Nva residue is the same. It is very interesting that the third position, which originally functions as a receptor interaction site with a positive charge, may function as a spacer to fix a peptide backbone to the conformation to exhibit great anti-platelet activity.

In the previous report,[31] we proposed a novel motif sequence, Pro-X1-X2-X3-Asp-X4, for anti-platelet activity. However, in the results of the present structure–activity relationship studies by replacing each residue of this motif sequence with L-form natural α-amino acids, the X1 and X3 positions showed narrow capacity for the amino acid substitution. Especially, the X3 position, which is originally a Gly residue, had extremely limited capacity for substitution such as alanine, proline, and arginine and allowed only a Gly residue for potent activity, suggesting that this position in the novel motif sequence still possesses the character of the Gly residue in the RGD sequence. Therefore, the motif sequence can be simplified to Pro-X1-X2-Gly-Asp-X4.

The results of the experiment in which the substitution of the X1 residue by an amino acid containing a large or acidic side chain resulted in complete loss of the anti-platelet activity and the substitution by an amino acid containing a small or a basic amino acid exhibited moderate activity indicate that the Ser residue at this position is important to exhibit potent activity. It is probable that a hydroxyl group of this Ser residue may be responsible for interaction with a receptor forming a hydrogen bond (Figure 2). On the other hand, an amino acid residue with a large side chain at this position may conformationally affect the interaction between the N-terminal Pro residue and the GpIIb/IIIa receptor and an acidic amino acid may also prevent interaction of the Pro residue with the receptor by undergoing ionic interaction with the N-terminal imino group of the Pro residue.

It is shown that the substitution of the residue at the X2 site by natural amino acids results in a slight decrease in activity (Table 5), suggesting that the side chain of an amino acid at this position may not greatly interfere with the interaction with the GpIIb/IIIa receptor. An amino acid with a relatively hydrophilic side chain or a small amino acid tends to decrease the activity, so that a large hydrophobic amino acid was suitable for increasing the activity due to the steric effect discussed above.

In the substitution of the C-terminal (X4) position, the results suggested that the C-terminal carboxylic acid is not so critical that another amino acid or a peptide chain can be added to the C-terminal without decreasing the activity. Therefore, it is expected that, if this motif

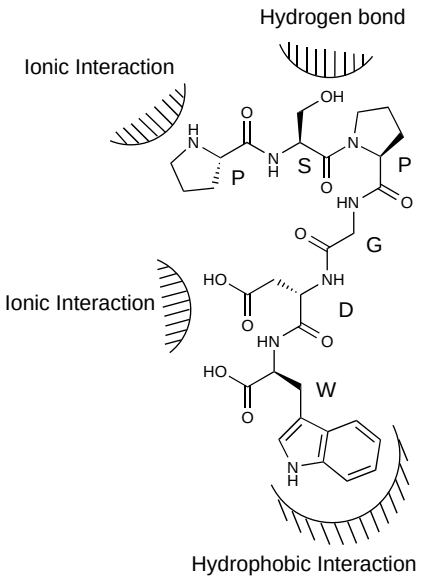

Figure 2. Schematic representation of Pro-Ser-X2-Gly-Asp-X4 pharmacophore.

sequence is inserted in the N-terminal of some biologically active peptides or proteins, the inserted motif sequence may function as a molecular missile which enables their specific targeting to activated platelets and/or megakaryocytes in which the functional GpIIb/IIIa is expressed.

According to these structure-activity relationship studies, it is suggested that one of the representative motif peptides, Pro-Ser-X2-Gly-Asp-X4 (X2: any L-form amino acid; X4: preferably aromatic amino acids), is a pharmacophore (Figure 2) that is responsible for their anti-platelet activity at submicromolar concentrations.

Conclusion

It is proposed that Pro-X1-X2-X3(Gly)-Asp-X4 is a newly discovered peptide motif which functions as a platelet aggregation inhibitor. These new motif peptides with no arginine residue, which exhibit potent binding activity and high specificity for GpIIb/IIIa, are provided as a useful tool in integrin research as well as agents in anti-thrombotic therapy.

Experimental

Peptide synthesis and purification

Peptides were synthesized by the solid phase method utilizing an Fmoc strategy[39] and 4-(benzyloxy)benzyl alcohol resin (Wang resin) (PE Applied Biosystems, Inc.). The protected amino acids were assembled sequentially by an automated Applied Biosystems instrument (Model 431A) employing a program supplied by the manufacturers for the Fast Moc™ procedure.[40] Side chain protected N^{α}-Fmoc amino acids were purchased from Watanabe Chemical or were synthesized by standard methods. Hydrolysis of the peptides for amino acid analyses was performed in 4 N MSA (containing 0.2% 3-(2-aminoethyl)indole) at 110°C for 24 h, and amino acids were analyzed with an automatic amino acid analyzer (L-8500, Hitachi). Mass spectra (fast-atom bombardment, low-resolution, glycerol matrix or electrospray ionization, methanol as the mobile phase) were analyzed with DX303 (JEOL) or Finnigan SSQ 7000, respectively. HPLC was carried out using a Jasco system (800 series) equipped with a UV/VIS detector and an integrator. The solvent system used for analytical HPLC was a binary system, water containing 0.1% TFA and acetonitrile containing the same TFA as the organic modifier, and the solvent program involved linear gradients as 5–40% acetonitrile over 35 min at a flow rate of 1 mL/min. The column used for analytical chromatography had dimensions of 4.6×250 mm (Wakosil-II 5C18 HG). HPLC on a semipreparative scale was performed with a reverse phase column (Waters, μBondasphere 19×150 mm, 10 μm, C-18) employing the same binary solvent system used for analytical HPLC at a flow rate of 17 mL/min on a Waters system (600E series). The analytical data for the purified peptides are given in Table 9. GRGDS was purchased from the Peptide Institute, Inc.

General method for peptide synthesis

The protected N^{α}-Fmoc amino acids (4 equiv) were sequentially coupled to the growing peptide chain on a Wang resin using Fast Moc™ procedure on an automated Applied Biosystem instrument. At the end of the synthesis, the N^{α}-Fmoc group was removed automatically as programmed. Acetylation of the N-terminal of the protected peptide resin was performed manually using acetic anhydride and 1-hydroxybenzotriazol (HOBT) in dimethylformamide (DMF) with the reaction time of 1 h. Each peptide-resin was dried in vacuo, and the peptide was then cleaved from the resin (0.25 mmol) using 1 M trimethylsilyl bromide-thioanisole[41] in TFA (10 ml) in the presence of 4.5% m-cresol and 2% ethanedithiol [in the case of peptide resins with Arg(Mtr)] for 1 h at 0 °C or using 20 mL of TFA containing thioanisole (1 mL), m-cresol (1 mL) and ethanedithiol (0.2 mL) (in other cases) for 2 h at room temperature. After removal of the resin with a glass filter, the filtrate was concentrated down by evaporation at room temperature and the residue was washed repeatedly with diethyl ether and then extracted with

Table 9. Peptide analytical data

Peptide no.	Amino acid analysis (cal) obtained[a,b]	MS		HPLC[c] K′ values	TLC[d] R_f values		
		[M + H]obsd	[M + H]calcd		A	B	C
1	Asp(1)1.00,Ser(1)0.97,Gly(1)1.06,Trp(1)0.92,Arg(1)1.01,Pro(1)1.09	717[e]	717	4.75	0.22	0.26	0.11
2	Asp(1)1.00,Ser(1)0.90,Gly(1)1.04,Arg(1)1.00,Pro(1)1.01,Trp(1)0.97	759[f]	759	5.79	0.29	0.70	0.24
3	Asp(1)1.00,Ser(1)0.93,Gly(1)1.05,Arg(1)1.00,Trp(1)0.93	731[e]	731	4.72	0.19	0.20	0.07
4	Asp(1)1.00,Ser(1)0.91,Gly(1)1.06,Arg(1)1.01,Trp(1)0.93	718[e]	718	6.74	0.41	0.69	0.28
5	Asp(1)1.00,Ser(1)0.94,Gly(1)1.06,Trp(1)0.97,Arg(1)1.02	731[e]	731	4.84	0.27	0.31	0.13
6	Asp(1)1.00,Ser(1)0.91,Gly(1)1.03,Pro(1)1.06,Trp(1)0.97	660[e]	660	6.24	0.41	0.70	0.28
7	Asp(1)1.00,Ser(1)0.93,Gly(1)1.05,Ala(1)1.05,Pro(1)1.09,Trp(1)1.05	632[e]	632	5.15	0.32	0.64	0.25
8	Asp(1)1.00,Ser(1)0.93,Gly(1)1.05,Val(1)1.04,Pro(1)1.09,Trp(1)1.01	660[f]	660	5.48	0.38	0.70	0.28
9	Asp(1)1.00,Ser(1)0.93,Gly(1)1.05,Leu(1)1.06,Pro(1)1.09,Trp(1)1.07	674[e]	674	6.71	0.42	0.68	0.26
10	Asp(1)1.00,Ser(1)0.97,Gly(1)1.04,Pro(1)1.10,Trp(1)0.97	674[e]	674	7.02	0.39	0.66	0.31
11	Asp(1)1.00,Ser(1)0.94,Gly(1)1.05,Ile(1)1.07,Pro(1)1.10,Trp(1)0.93	674[e]	674	6.72	0.41	0.68	0.29
12	Asp(1)1.00,Ser(1)0.96,Gly(1)1.06,Pro(1)0.95,Trp(1)0.96	674[e]	674	6.68	0.41	0.63	0.25
13	Asp(1)1.00,Ser(1)0.94,Gly(1)0.92,Pro(1)1.03,Trp(1)0.97	674[e]	674	6.63	0.45	0.68	0.29
14	Asp(1)1.00,Ser(1)0.95,Gly(1)1.07,Pro(1)1.09,Trp(1)0.94	700[e]	700	7.57	0.46	0.72	0.33
15	Asp(1)1.00,Ser(1)0.94,Gly(1)1.05,Pro(1)1.08,Trp(1)1.07	694[e]	694	6.74	0.41	0.67	0.30
16	Asp(1)1.00,Ser(1)0.91,Gly(1)1.04,Pro(2)2.05,Trp(1)0.99	658[e]	658	5.31	0.35	0.56	0.24
17	Asp(1)1.00,Ser(1)0.96,Gly(1)1.05,Pro(1)0.95,Trp(1)0.94	674[e]	674	4.76	0.31	0.44	0.13
18	Asp(1)1.00,Ser(1)0.93,Gly(1)1.05,Pro(1)1.00,Trp(1)1.05	656[e]	656	5.36	0.32	0.58	0.23
19	Asp(1)1.00,Ser(1)0.91,Gly(1)1.04,Pro(1)0.97,Trp(1)0.95	676[f]	676	5.74	0.39	0.49	0.26
20	Asp(1)1.00,Ser(1)0.90,Gly(1)1.05,Pro(1)0.96,Trp(1)1.04	704[f]	704	6.80	0.45	0.68	0.31
21	Asp(1)1.00,Ser(1)0.93,Gly(1)1.04,Pro(1)0.98,Trp(1)0.96	764[f]	764	8.21	0.46	0.69	0.33
22	Asp(1)1.00,Ser(1)0.95,Gly(1)1.07,Trp(1)1.00,Pro(2)2.15	658[e]	658	5.28	0.36	0.55	0.26
23	Asp(1)1.00,Ser(1)0.93,Gly(1)1.06,Pro(1)1.04,Trp(1)1.03	644[e]	644	4.96	0.30	0.57	0.22
24	Asp(1)1.00,Ser(1)0.88,Gly(1)1.06,Pro(1)1.11,Trp(1)0.94	672[e]	672	6.13	0.39	0.63	0.27
25	Asp(1)1.00,Ser(1)0.93,Gly(1)1.06,Pro(2)2.04,Trp(1)1.01	700[f]	700	6.67	0.42	0.68	0.26
26	Ser(1)0.92,Glu(1)1.00,Gly(1)1.06,Pro(2)2.05,Trp(1)1.02	672[e]	672	5.46	0.41	0.67	0.26
27	Ser(1)0.89,Gly(1)1.00,Ala(1)1.01,Pro(2)1.96,Trp(1)1.03	614[f]	614	6.38	0.42	0.59	0.24
28	Asp(1)1.00,Gly(1)1.07,Ala(1)1.08,Pro(2)2.18,Trp(1)1.05	642[e]	642	5.64	0.35	0.56	0.22
29	Asp(1)1.00,Gly(2)2.06,Pro(2)1.91,Trp(1)0.97	628[e]	628	5.84	0.33	0.61	0.21
30	Asp(1)1.00,Gly(1)1.02,Ile(1)1.03,Pro(2)1.98,Trp(1)0.99	684[e]	684	6.68	0.45	0.66	0.33
31	Asp(1)1.00,Gly(1)0.97,Met(1)0.92,Pro(2)1.90,Trp(1)1.02	702[f]	702	7.03	0.42	0.66	0.26
32	Asp(1)1.00,Gly(1)1.03,Phe(1)1.02,Pro(2)1.99,Trp(1)1.00	718[f]	718	7.33	0.47	0.69	0.35
33	Asp(1)1.00,Gly(1)1.03,Arg(1)1.00,Pro(2)1.92,Trp(1)0.97	727[f]	727	5.28	0.28	0.24	0.18
34	Asp(1)1.00,Gly(1)1.04,His(1)1.04,Pro(2)1.93,Trp(1)1.04	708[f]	708	5.21	0.23	0.22	0.18
35	Asp(1)1.00,Gly(1)1.08,Tyr(1)1.05,Pro(2)2.00,Trp(1)0.97	734[f]	734	6.32	0.38	0.68	0.33
36	Asp(1)1.00,Glu(1)0.99,Gly(1)1.03,Pro(2)2.10,Trp(1)0.98	700[f]	700	5.52	0.30	0.53	0.23
37	Asp(1)1.00,Ser(2)1.91,Gly(1)1.07,Pro(1)0.94,Trp(1)0.96,	648[f]	648	4.67	0.27	0.61	0.18
38	Asp(1)1.00,Ser(1)0.94,Gly(1)1.08,Met(1)1.03,Pro(1)0.99,Trp(1)0.94	692[e]	692	6.35	0.42	0.64	0.27
39	Asp(1)1.00,Ser(1)0.93,Gly(1)1.04,Phe(1)1.02,Pro(1)1.07,Trp(1)1.05	708[e]	708	7.29	0.46	0.68	0.32
40	Asp(1)1.00,Ser(1)0.96,Gly(1)1.06,Lys(1)1.05,Pro(1)1.07,Trp(1)0.94	689[e]	689	4.39	0.18	0.21	0.08
41	Asp(1)1.00,Ser(1)0.98,Gly(1)1.06,His(1)1.06,Pro(1)1.05,Trp(1)1.03	698[e]	698	4.65	0.17	0.22	0.13
42	Asp(1)1.00,Ser(1)0.92,Gly(1)1.06,Tyr(1)1.11,Pro(1)1.09,Trp(1)0.97	724[e]	724	6.16	0.40	0.69	0.32
43	Asp(1)1.00,Ser(1)0.95,Glu(1)1.01,Gly(1)1.04,Pro(1)0.99,Trp(1)1.06	690[f]	690	5.03	0.24	0.45	0.24
44	Asp(1)1.00,Ser(1)0.94,Ala(1)1.03,Pro(2)2.15,Trp(1)1.08	672[e]	672	5.88	0.34	0.65	0.22
45	Asp(1)1.00,Ser(2)1.92,Pro(2)2.18,Trp(1)0.92	688[e]	688	5.54	0.32	0.63	0.21
46	Asp(1)1.00,Ser(1)1.05,Thr(1)1.07,Pro(2)2.12,Trp(1)0.99	702[e]	702	5.63	0.32	0.59	0.24
47	Asp(1)1.00,Ser(1)0.96,Pro(3)3.23,Trp(1)1.04	698[e]	698	5.52	0.28	0.42	0.22
48	Asp(1)1.00,Ser(1)0.96,Val(1)0.99,Pro(2)2.15,Trp(1)0.90	700[e]	700	6.40	0.38	0.53	0.19
49	Asp(1)1.00,Ser(1)0.99,Met(1)1.00,Pro(2)1.98,Trp(1)1.05	732[f]	732	6.88	0.43	0.66	0.28
50	Asp(1)1.00,Ser(1)0.99,Ile(1)1.06,Pro(2)2.13,Trp(1)1.01	714[f]	714	7.06	0.43	0.67	0.32
51	Asp(1)1.00,Ser(1)0.93,Phe(1)1.05,Pro(2)2.14,Trp(1)0.90	748[f]	748	8.10	0.44	0.67	0.35
52	Asp(1)1.00,Ser(1)0.97,Arg(1)1.04,Pro(2)2.10,Trp(1)0.95	757[f]	757	5.16	0.26	0.25	0.14
53	Asp(1)1.00,Ser(1)0.98,His(1)1.05,Pro(2)2.05,Trp(1)0.92	739[f]	738	5.05	0.24	0.23	0.14
54	Asp(1)1.00,Ser(1)0.96,Tyr(1)1.13,Pro(2)2.11,Trp(1)0.97	764[e]	764	6.66	0.36	0.68	0.29
55	Asp(1)1.00,Ser(1)0.96,Glu(1)1.03,Pro(2)2.06,Trp(1)1.09	730[f]	730	5.67	0.29	0.55	0.23
56	Asp(1)1.00,Ser(1)0.93,Gly(2)1.81,Pro(2)2.10	529[f]	529	1.14	0.12	0.21	0.04

(*continued*)

Table 9—*contd*

57	Asp(1)1.00,Ser(2)1.75,Gly(1)1.02,Pro(2)2.09	559[f]	559	0.63	0.16	0.21	0.05
58	Asp(1)1.04,Ser(1)0.94,Gly(1)1.00,Ile(1)0.98,Pro(2)1.90	586[f]	585	4.20	0.33	0.38	0.22
59	Asp(1)1.00,Ser(1)0.89,Gly(1)0.99,Met(1)0.97,Pro(2)1.86	603[f]	603	3.34	0.28	0.31	0.11
60	Asp(1)1.00,Ser(1)0.89,Gly(1)0.99,Pro(2)1.98,Phe(1)0.94	619[f]	619	4.81	0.30	0.40	0.11
61	Asp(1)1.00,Ser(1)0.89,Gly(1)1.01,Arg(1)0.97,Pro(2)2.01	628[f]	628	1.19	0.13	0.11	0.03
62	Asp(1)1.00,Ser(1)0.88,Gly(1)1.00,Tyr(1)1.05,Pro(2)1.95	635[f]	635	3.61	0.29	0.37	0.13
63	Asp(1)1.02,Ser(1)0.92,Glu(1)0.96,Gly(1)1.00,Pro(2)1.91	601[f]	601	1.55	0.13	0.26	0.06
64	Asp(1)1.00,Ser(1)0.96,Gly(1)1.05,Trp(1)0.97,Pro(2)2.06	657[f]	657	4.59	0.38	0.45	0.16
65	Asp(1)1.00,Ser(1)0.96,Gly(1)1.04,Arg(1)1.00,Pro(2)2.02,Trp(1)1.06	814[f]	814	4.62	0.28	0.22	0.14

[a]We did not look for the presence of non-natural amino acid. That was confirmed by MS. Hydrolysis of the peptides for amino acid analyses was performed in 4 N MSA (containing 0.2% 3-(2-aminoethyl)indole) at 110 °C for 24 h.
[b]Theoretical values are in parentheses.
[c]Wakosil-II 5 C_{18} HG column (4.6×250 mm) eluted in a linear gradient. (5 to 40% acetonitrile over 35 min at a flow rate of 1.0 mL/min).
[d]TLC systems: A = nBuOH:pyridine:AcOH:H_2O 4:1:1:2; B = nBuOH:AcOH:AcOEt:H_2O 1:1:1:1; C = nBuOH:AcOH:H_2O 4:1:5 (upper phase).
[e]Fast atom bombardment (FAB)-MS.
[f]Electrospray ionization (ESI)-MS.

10% acetic acid (10 mL). This solution with the crude peptide was then purified by semipreparative reverse phase HPLC under the conditions described above to yield a white powder after lyophilization. The structure was corroborated by the results of amino acid analysis and mass spectrometry, and the purity of each product was assessed by analytical HPLC and TLC.

In vitro platelet aggregation

Platelet aggregation studies were performed in platelet-rich plasma (PRP) obtained from human volunteers. Blood was drawn into plastic syringes containing 1/10 volume of 3.8% trisodium citrate. PRP was prepared by centrifugation of citrated whole blood at 160×*g* for 15 min at room temperature. PRP was removed and the platelet count was determined. Platelet-poor plasma was obtained by centrifugation of the remaining blood at 2000×*g* for 15 min. Saline or peptide solution of various concentrations was added to PRP at 37 °C 1 min prior to the initiation of platelet aggregation. Platelet aggregation was initiated with 5 μg/mL collagen and the aggregation was measured in an aggregometer (NBS Hematracer-601, Nikoh Bioscience Co., Ltd., Tokyo) as an increase in light transmission. Platelet aggregation is presented as the percent inhibition of the rate of platelet aggregation compared to control samples, and IC_{50} values were calculated from dose-inhibition curves. Throughout the platelet aggregation assay, GRGDS peptide was used as a reference compound, and we confirmed that the IC_{50} values of this peptide did not significantly vary with PRPs from different blood donors (470 ± 23 μM; mean ± sem from five different donors). Therefore, the absolute IC_{50} values are listed in each table.

Solid phase binding assay

The inhibitory effects of the peptides on integrin-ligand interactions were evaluated using a competitive enzyme-linked immunosorbent assay (ELISA).[19] Briefly, for vitronectin-αvβ3 ELISA, 96-well microtiter plates were coated overnight at room temperature with 10 mg/mL human vitronectin (Iwaki Glass Co., Ltd., Tokyo), and the plates were washed with TBS containing 0.05% Tween 20. αvβ3 solution, whose preparation was described in our previous report,[31] was added to each well, and then peptide solutions of various concentrations were added to each well. The plates were incubated at room temperature for 2 h and washed with TBS containing 0.05% Tween 20. Bound receptor was detected using anti-human αv rabbit polyclonal antibody (Chemicon Int., Inc.) and affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase.

Fibrinogen-GpIIb/IIIa ELISA was performed using a similar protocol except that the microtiter plates were coated with 10 mg/mL human fibrinogen (Sigma), and GpIIb/IIIa solution, whose preparation was also described in our previous report,[31] and anti-human αII mouse monoclonal antibody was used.

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