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Design and Synthesis of Novel Cyclic RGD-Containing Peptides as Highly Potent and Selective Integrin $\alpha_{IIb}\beta_3$ Antagonists

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Utilizing conformational constraints in conjunction with various structural considerations, we have synthesized a series of cyclic disulfide peptides that are highly potent and selective antagonists for the platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa). The affinities of the peptides for $\alpha_{IIb}\beta_3$ were determined by platelet aggregation assays and an $\alpha_{IIb}\beta_3$ ELISA. Their affinities for $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins were also determined in respective ELISA assays. Structure–activity relationship studies suggest that R-G-D-Ar-R (Ar = hydrophobic residue) is the essential pharmacophore that is responsible for their high $\alpha_{IIb}\beta_3$ binding affinity, very high selectivity, and distinct biological properties. One of these analogues, TP9201, has been shown to inhibit platelet-mediated thrombus formation without associated prolongation of template bleeding time. The arginine residue adjacent the carboxy terminus of the R-G-D-Ar sequence could function as the biological effector element that determines this distinct and unexpected biological property.

Introduction

Initial events in thrombus formation frequently entail the activation of platelets by thrombogenic surfaces and their subsequent aggregation.[1] Adhesion and aggregation of platelets is mediated by adhesive proteins that interact with the platelet membrane glycoprotein complex $\alpha_{IIb}\beta_3$ at the platelet surface.[2] Platelet $\alpha_{IIb}\beta_3$ is a member of the family of cell adhesion receptors, called integrins.[3-5] It has been shown that, like several of the integrins,[4] $\alpha_{IIb}\beta_3$ on activated platelets can bind to an Arg-Gly-Asp (RGD)[6] tripeptide sequence[7-9] in several proteins: fibrinogen, fibronectin, von Willebrand factor, and vitronectin.[8-10] Inhibition of the binding of fibrinogen to $\alpha_{IIb}\beta_3$ via molecules having structures based on the RGD tripeptide sequence is a promising approach for the inhibition of platelet aggregation and subsequent thrombus formation because it targets the final step in the aggregation process.

The antithrombotic activity of many molecules that inhibit the $\alpha_{IIb}\beta_3$–fibrinogen interaction has been assessed *in vitro* and *in vivo*. Most of these compounds fall into four categories: RGD-based peptides (small linear and cyclic peptides containing the RGD sequence or its equivalent),[11-17] snake venom peptides,[18-22] monoclonal antibodies raised against $\alpha_{IIb}\beta_3$,[23-25] and non-peptide fibrinogen receptor antagonists that mimic the RGD tripeptide sequence.[26-30]

Small linear and cyclic peptides containing the RGD sequence or its equivalent have been demonstrated to inhibit fibrinogen binding to $\alpha_{IIb}\beta_3$ and, thereby, to prevent platelet aggregation.[11-17] Many small potent $\alpha_{IIb}\beta_3$ antagonists have been developed. So far, little information about integrin selectivity has been published and very little is known about the actual structure and activity/selectivity relationship.[31] Selectivity is an important concern because RGD-directed integrin receptors are widely distributed and participate in various physiological and pathological processes.[32] It has also been reported that at least one of these potent synthetic peptides causes marked prolongation of template bleeding time at the

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intravenous doses that are required for effective inhibition of *in vivo* thrombus formation or *ex vivo* platelet aggregation.[33]

Many RGD-containing viper venom proteins have been isolated and characterized, including echistatin,[11] kistrin[15], trigramin[13] and bitistatin.[14] These peptides, which contain one or more RGD sequences, are 47–83 amino acids in length with reported IC_{50} values of 1–3 nM for the inhibition of fibrinogen binding to $\alpha_{IIb}\beta_3$. They inhibit platelet aggregation with IC_{50} values in the range 110–550 nM. Several recent NMR studies of echistatin have been reported.[34–36] The RGD sequence is located at the tip of a flexible loop; thus its conformation is ill-defined in these proteins. Because snake venom proteins are nonspecific integrin inhibitors, they can cause unwanted bleeding problems.

Monoclonal antibodies against the $\alpha_{IIb}\beta_3$ have been analyzed for antithrombotic activity. Some have been shown to have sequences in their hypervariable regions related to RGD.[37–39] The $F(ab')_2$ fragments of 7E3, a monoclonal antibody that binds β_3 and inhibits platelet aggregation, have been shown to inhibit platelet rich thrombus formation, to accelerate coronary thrombolysis, and to prevent reocclusion after thrombolysis.[17] However, a monoclonal antibody derived therapeutic has a potential disadvantage; it has been reported that it can cause marked prolongation of bleeding time at the dose required to abolish *ex vivo* platelet aggregation.[25]

In addition, several groups have reported the discovery of non-peptide, RGD mimics that function as inhibitors of platelet aggregation.[26–30] Clinical success with these glycoprotein $\alpha_{IIb}\beta_3$ inhibitors will depend, as it will with all types of inhibitors, on their not causing unwanted bleeding or hemorrhagic problems at doses providing effective inhibition of platelet aggregation.

Because of the medical and biological importance of platelet adhesion, it is important to design peptides for use as antagonists that bind to the $\alpha_{IIb}\beta_3$ receptor specifically and with a high affinity. Attempts to design biologically potent and receptor-selective peptides require the identification of residues necessary for binding and the design of elements that will ensure that the conformation necessary for binding is heavily populated. One approach for the design of very selective ligands involves the incorporation of conformational restrictions.[40] The use of conformational constraints has proven to be highly effective in obtaining highly potent and receptor-selective peptide ligands.[41] We have shown that synthetic peptides containing the RGD sequence can be designed to exhibit varying integrin specificities by the use of conformational constraints.[42] Presumably, RGD-containing analogues that exhibit such properties have been constrained to a conformation or a family of conformations that closely resembles that required for productive peptide/receptor interactions.

The arginine, glycine, and aspartic acid residues in the RGD tripeptide sequence are essential for activity, and the arginine side chain and the aspartic side chain are critical for binding. One hypothesis for the specificity of the interactions between the receptors and the RGD-containing peptides is that the RGD tripeptide can provide all the information needed for specificity, and the role of the surrounding sequences would be to force the RGD tripeptide into an appropriate conformation for the receptor to recognize. This hypothesis assumes that specific "bioactive conformations" of synthetic RGD peptides are binding to different receptors and the activity of the differently constrained cyclic peptides can be rationalized in terms of relative orientations of the Arg and Asp side chains and backbone elements of the RGD sequences. Several recent NMR studies have attempted to characterize the RGD conformations in bioactive peptides.[43–45]

Alternatively, the nature of residues flanking the RGD sequence could influence receptor affinity, receptor selectivity, and other biological properties. The side chains and backbone elements of residues flanking the RGD sequence may play very important roles in the recognition and may function as critical biological effector elements. To investigate the contribution of individual amino acids, we have previously prepared a series of cyclic peptides, with different residues adjacent to the RGD tripeptide sequence.[46] We have shown that G(Pen)GHRGDLRCA exhibits very low affinities for the $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins, while it inhibits platelet aggregation with an IC_{50} of 13.7 μM. This suggests that the G(Pen)GHRGDLRCA peptide more closely mimics the RGD structure preferred by the $\alpha_{IIb}\beta_3$ binding site.[46] In this molecule, conformational restriction is induced by disulfide bond formation between the penicillamine (β,β-dimethylcysteine) residue in position -3[47] and cysteine residue in position 5 and by the geminal dimethyl groups of the penicillamine residue.[48] Structure–activity relationship studies[49] were undertaken in order to improve the binding affinity to $\alpha_{IIb}\beta_3$ as well as increase $\alpha_{IIb}\beta_3$ selectivity (versus $\alpha_5\beta_1$ and $\alpha_v\beta_5$) and to probe which selected molecular features contribute to distinct biological properties.

Chemistry. All the peptides were synthesized using a solid-phase method.[50,51] N^{α}-*tert*-Butyloxycarbonyl (Boc) protection was used on all amino acids. Anhydrous liquid HF was used for the final deprotection. The crude peptides were purified by preparative HPLC. All the peptides were characterized by FABMS, AAA, and analytical HPLC. The purified peptides were at least 98% pure.

Assay Systems. Four different assays were used to assess the biological activity and selectivity of the peptides. A platelet aggregation assay directly measured the effects of peptides on platelet function. The platelet aggregation in human platelet-rich citrated plasma (PRP) was induced by 10 μM ADP. The progress and the extent of the platelet aggregation reaction was monitored by measuring transparency of the platelet suspension in an aggregometer. Differences among platelet donors or day-to-day variation of platelets from the same donor may cause IC_{50} values of peptides to vary. The IC_{50} was adjusted by the ratio between the IC_{50} value of a standard determined in every experiment. The affinities of peptides for $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_5$ were measured by respective ELISA assays as described.[52,53]

Results and Discussion

Effects of Pen Substitutions (Table 1). To investigate the effect on $\alpha_{IIb}\beta_3$ potency and selectivity of altering the chirality at the amino-terminal L-Pen in the prototype peptide **1**, the D-Pen-containing analogue **2** was synthesized. Analogue **2** was approximately 2-fold more potent than the L-Pen-containing analogue **1** in the $\alpha_{IIb}\beta_3$ assay. The Pen residue with the D configuration at the amino terminus had no significant effect on the selectivity. The situation is similar for analogues **3** and **4**. The D-Pen-

Table 1. Modifications of G(Pen)GHRGDLRCA

no.	peptide[a]	platelet aggregation human PRP/ADP IC_{50} (μM)	IC_{50} (μM) $\alpha_{IIb}\beta_3$ ELISA	$\alpha_5\beta_1$ ELISA	$\alpha_v\beta_5$ ELISA
1	G(Pen)GHRGDLRCA	13.7	3.44	3.44	8.26
2	G(D-Pen)GHRGDLRCA	7.1	0.15	1.38	5.27
3	R(Pen)GHRGDLRCR	9.6	0.04	nt[b]	4.93
4	R(D-Pen)GHRGDLRCR	4.1	0.15	0.10	10.00
5	R(Pmc)GHRGDLRCR	3.0	0.40	5.19	1.58
6	(Pmp)GHRGDLRCA	5.9	0.02	0.83	10.00
7	R(Tmc)GHRGDLRCR	4.1	0.03	1.20	0.34

[a] Non-natural amino acid abbreviations are given in ref 6. [b] Not tested.

containing analogue 4 was 2-fold more potent in the aggregation assay than the L-Pen-containing analogue 3. We also examined the effects of additional conformational constraint in the side chains of the amino-terminal Pen, by evaluating amino-terminal Pmp[54] and Pmc[55,56] residues. Both contain the more lipophilic and conformationally restricting β,β-pentamethylene at the β-carbon atom of the side chain. These residues have previously been used to further restrict the conformational freedom of other disulfide-bridged peptides leading to highly constrained ring systems.[40] Analogue 5, which contains D,L-Pmc at position -3, was prepared by employing Boc-D,L-Pmc(4-MeBzl). Analogue 5 was found to be 3-fold more potent than analogue 3 in the aggregation assay. On the other hand, the substitution of Pen with an amino-terminal Pmp group in analogue 6 resulted in a 2-fold increase in potency over analogue 1. The structure of this analogue suggests that an exocyclic residue at the amino terminus is not necessary for potency. We also evaluated the Tmc residue, which contains β,β-tetramethylene at the β-carbon atom of the side chain, as a substitution for the amino-terminal Pen residue. Analogue 7, which contains D,L-Tmc at position -3, was prepared by employing Boc-D,L-Tmc(4-MeBzl). The mixture of diasteromers was 2-fold more potent than analogue 3 in the platelet aggregation assay.

This series of analogues demonstrated that the substitution of Pmc for Pen is effective in obtaining more potent analogues. Altering the chirality at the amino terminal Pen residue had only a modest effect on the selectivity.

Effects of Substitutions at Position 3 (Table 2). A subsequent series of analogues was prepared on the basis of the sequence of the potent and selective analogue 5, R(Pmc)GHRGDLRCR. The effect of increased hydrophobicity at position 3 of analogue 5 was investigated by the incorporation of a series of hydrophobic amino acids at this position. It was found that the substitution of aromatic hydrophobic residues (Phe, Tyr) for Leu is very effective in obtaining more potent analogues. For example, the substitution of Leu with Phe resulted in about a 5-fold increase in platelet aggregation potency in analogue 9. The effect of para substitution of the aromatic ring in the Phe residue was investigated by the substitution of Phe with *p*-I-Phe and *p*-Cl-Phe. These two analogues (12 and 13) exhibited similar platelet aggregation potencies as that of analogue 9. The similar potency of these analogues implied that the aromatic nucleus of the Phe residue interacts with a region of $\alpha_{IIb}\beta_3$ that is capable of accepting a range of electronegativities. When our initial results showed promise, we expanded the study to include the following additional hydrophobic residues at position 3: O-Me-Tyr, O-*n*-butyl-Tyr,[57] Phg, Hpa, and 2-Nal. These analogues (11, 14, 15, 16, and 17) also exhibited similar platelet aggregation potencies as that of analogue 9. The most potent analogue of this series with respect to inhibition of platelet aggregation, analogue 14, contains one of the most hydrophobic aromatic side chains in this series. The high potency of these analogues implied that these hydrophobic residues interact with a region of $\alpha_{IIb}\beta_3$ that is capable of accepting a range of molecular volumes. Surprisingly, the substitution of Leu with a shorter aliphatic residue (Val) also resulted in a 4-fold increase in potency in analogue 8. This result suggests that the contribution of the β-methyl group of valine may be as significant as the aromatic group for the interaction with the hydrophobic region of $\alpha_{IIb}\beta_3$.

Effects of Substitutions in Position -2 (Table 3). Next, we examined the effect of the substitution of the Gly residue at position -2. Single amino acid substitutions (Ser, Asp, Ile, Asn, and Arg) were introduced into analogue 11, which is highly potent in platelet aggregation assay. We found that substitution of Ser, Ile, Asn, and Arg for Gly produced analogues (19, 20, 21, and 22) with similar platelet aggregation potencies and ELISA selectivities. However, the substitution of Asp for Gly (analogue 18) led to a 4-fold decrease in potency.

Effects of Substitutions at Position -1 (Table 4). We also examined the effect of substitution for His at position -1. Single amino acid substitutions (D-His, Tyr, D-Tyr, and Pro) were introduced into analogue 11, which afforded a series of analogues (23, 24, 25, and 26). We found that these substitutions had little influence on the platelet aggregation inhibitory potency of analogue 11. The study with peptides 23–26 suggests that His is not interacting with $\alpha_{IIb}\beta_3$ through hydrogen bonding or by other interactions characteristic of the aromatic ring. The substitution of His with Pro did not lead to a decrease in potency.

Effects of the Use of Pro at Position -1 in Combination with Hydrophobic Amino Acids at Position 3 (Table 5). It has been shown (Table 1) that an exocyclic Arg residue at the amino terminus is not necessary for potency. We reasoned that the exocyclic Arg residue at the carboxyl terminus is also probably not required for potency. The subsequent series of analogues were prepared on the basis of the sequence of analogue 26, and the Arg residues at the N-terminal and C-terminal were replaced with acetyl and amide groups, respectively. The substitution of Gly at position -2 of analogue 26 with Ile and the replacement of the Pmc residue with Cys resulted in a 3–4-fold increase in platelet aggregation potency in analogue 27, Ac-CIPRGD(Y-OMe)RC-NH_2, without affecting the potency in the $\alpha_5\beta_1$ and $\alpha_v\beta_5$ assays. In general, the substitution of the Ile for Gly is very effective in obtaining more potent analogues (compare Tables 4 and 5).

Proline is a well-known means of inducing conformational constraints into peptides.[58,59] The substitution of Ile with Asn at position -2 had little effect on the platelet aggregation potency. The subsequent series of analogues was based on the sequence of the potent and selective analogue 27, Ac-CIPRGD(Y-OMe)RC-NH_2 and analogue 32, AC-CNPRGD(Y-OMe)RC-NH_2. The use of Pro at position -1 in combination with hydrophobic amino acids at position 3 yields a series of highly potent and specific $\alpha_{IIb}\beta_3$ antagonists. It was found that substitution of Tyr for Tyr(OMe) resulted in a 2-fold decrease in potency.

Table 2. Modifications of R(Pmc)GHRGDLRCR

no.	peptide[a,b]	platelet aggregation human PRP/ADP IC_{50} (μM)	IC_{50} (μM) $\alpha_{IIb}\beta_3$ ELISA	$\alpha_5\beta_1$ ELISA	$\alpha_v\beta_5$ ELISA
5	R(Pmc)GFRGDLRCR	3.00	0.40	5.19	1.58
8	R(Pmc)GHRGDVRCR	0.69	0.15	2.30	2.00
9	R(Pmc)GHRGDFRCR	0.62	0.04	8.70	10.00
10	R(Pmc)GHRGDYRCR	0.97	0.15	10.00	7.85
11	R(Pmc)GHRGD(Y-OMe)RCR	0.56	0.02	0.98	1.05
12	R(Pmc)GHRGD(*p*-I-F)RCR	0.70	0.01	4.19	4.03
13	R(Pmc)GHRGD(*p*-Cl-F)RCR	0.73	0.02	0.87	15.30
14	R(Pmc)GHRGD(Y-O-*n*-butyl)RCR	0.35	0.03	6.60	2.20
15	R(Pmc)GHRGD(Phg)RCR	0.68	0.02	1.23	0.79
16	R(Pmc)GHRGD(Hpa)RCR	0.60	0.02	1.81	4.25
17	R(Pmc)GHRGD(2-Nal)RCR	0.44	0.07	7.72	10.00

[a] Non-natural amino acid abbreviations are given in ref 6. [b] Peptide 5 and peptides 8–17 were prepared by employing Boc-D,L-Pmc(4-MeBzl).

Table 3. Modifications of R(Pmc)GHRGD(Y-OMe)RCR

no.	peptide[a,b]	platelet aggregation human PRP/ADP IC_{50} (μM)	IC_{50} (μM) $\alpha_{IIb}\beta_3$ ELISA	$\alpha_5\beta_1$ ELISA	$\alpha_v\beta_5$ ELISA
11	R(Pmc)GHRGD(Y-OMe)RCR	0.56	0.02	0.98	1.05
18	R(Pmc)DHRGD(Y-OMe)RCR	2.75	0.21	9.20	7.20
19	R(Pmc)SHRGD(Y-OMe)RCR	0.95	0.12	>10.00	6.60
20	R(Pmc)IHRGD(Y-OMe)RCR	0.83	0.06	6.40	9.90
21	R(Pmc)NHRGD(Y-OMe)RCR	0.99	0.09	4.70	6.90
22	R(Pmc)RHRGD(Y-OMe)RCR	0.44	0.07	7.72	6.20

[a] Non-natural amino acid abbreviations are given in ref 6. [b] Peptides 18–22 were prepared by employing Boc-D,L-Pmc(4-MeBzl).

Table 4. Modifications of R(Pmc)GHRGD(Y-OMe)RCR

no.	peptide[a,b]	platelet aggregation human PRP/ADP IC_{50} (μM)	IC_{50} (μM) $\alpha_{IIb}\beta_3$ ELISA	$\alpha_5\beta_1$ ELISA	$\alpha_v\beta_5$ ELISA
11	R(Pmc)GHRGD(Y-OMe)RCR	0.56	0.02	0.98	1.05
23	R(Pmc)G(DH)RGD(Y-OMe)RCR	0.40	0.01	6.8	nt[c]
24	R(Pmc)GYRGD(Y-OMe)RCR	0.85	0.03	8.5	8.80
25	R(Pmc)G(DY)RGD(Y-OMe)RCR	0.52	0.01	10.0	10.00
26	R(Pmc)GPRGD(Y-OMe)RCR	0.54	0.01	7.2	1.70

[a] Non-natural amino acid abbreviations are given in ref 6. [b] Peptides 23–26 were prepared by employing Boc-D,L-Pmc(4-MeBzl). [c] Not tested.

Table 5. Modifications of Ac-CIPRGD(Y-OMe)RC-NH_2

no.	peptide[a]	platelet aggregation human PRP/ADP IC_{50} (μM)	IC_{50} (μM) $\alpha_{IIb}\beta_3$ ELISA	$\alpha_5\beta_1$ ELISA	$\alpha_v\beta_5$ ELISA
27	Ac-CIPRGD(Y-OMe)RC-NH_2	0.17	0.003	4.30	5.80
28	Ac-CIPRGDYRC-NH_2	0.33	0.003	4.50	>10.00
29	Ac-CIPRGDFRC-NH_2	0.45	0.014	2.10	10.00
30	Ac-CIPRGD(Y-O-*n*-butyl)RC-NH_2	0.48	0.016	6.00	>10.00
31	Ac-CIPRGD(*p*-NO_2-F)RC-NH_2	0.21	0.430	1.20	4.30
32	Ac-CNPRGD(Y-OMe)RC-NH_2	0.22	0.029	8.20	4.70
33	Ac-CNPRGD(Y-OEt)RC-NH_2	0.22	0.004	10.00	8.40
34	Ac-CNPRGD(Y-O-*n*-butyl)RC-NH_2	0.10	0.006	1.50	10.00
35	Ac-CNPRGD(*p*-NO_2-F)RC-NH_2	0.84	0.017	4.79	4.80

[a] Non-natural amino acid abbreviations are given in ref 6.

This result suggested that the *O*-methyl group of Tyr(OMe) may be important for the interaction with the hydrophobic region of $\alpha_{IIb}\beta_3$. The most potent analogue of this series is analogue **34**, with an IC_{50} in platelet aggregation of 0.1 μM. This analogue is about 150 times more potent than analogue **1** and exhibits a much higher selectivity for $\alpha_{IIb}\beta_3$.

This series of analogues demonstrated that the use of Pro at position −1 in combination with hydrophobic amino acids at position 3 is very effective in obtaining highly potent analogues.

Examination of the data for all the peptides in Tables 1–5 reveals a generally good correlation between the order of the IC_{50} values in the $\alpha_{IIb}\beta_3$ ELISA assay and the order in the platelet aggregation assay. A few peptides exhibit striking differences in potency between platelet aggregation and the $\alpha_{IIb}\beta_3$ ELISA. It may be the case, however, that the ELISA assay is more sensitive to differences in peptide structure. In this regard, the interaction of peptides with receptors embedded in the membrane on the platelet surface may be more complex than the interaction of peptides with a soluble, purified receptor.

Effects of Cys Substitutions on Analogue 32 (Table 6). The subsequent series of analogues were prepared based on the sequence of analogue **32**. Analogue **32** was found to be more potent than analogue **27** *in vivo* (J. Tschopp, unplublished data). We examined the effect of the substitution of the amino-terminal Cys residue with other β,β-dialkylcysteines and their desamino derivatives. Replacing the Cys residue with the more lipophilic and

Table 6. Modifications of Ac-CNPRGD(Y-OMe)RC-NH_2

no.	peptide[a]	platelet aggregation human PRP/ADP IC_{50} (μM)	IC_{50} (μM) $\alpha_{IIb}\beta_3$ ELISA	$\alpha_5\beta_1$ ELISA	$\alpha_v\beta_5$ ELISA
32	Ac-CNPRGD(Y-OMe)RC-NH_2	0.22	0.029	8.20	4.70
36	Ac-(D-Pen)NPRGD(Y-OMe)RC-NH_2	0.17	0.001	6.80	6.70
37	Ac-(Pmc)NPRGD(Y-OMe)RC-NH_2	0.15	0.016	>10.00	2.34
38	(Pmp)NPRGD(Y-OMe)RC-NH_2	0.17	0.031	5.10	4.90
39	(Mpr)NPRGD(Y-OMe)RC-NH_2	0.17	0.015	6.00	3.00
27	Ac-CIPRGD(Y-OMe)RC-NH_2	0.17	0.003	4.30	5.80
40	Ac-(D-Pen)IPRGD(Y-OMe)RC-NH_2	0.28	0.002	4.30	4.30

[a] Non-natural amino acid abbreviations are given in ref 6.

Table 7. Modification of Ac-CNPRGD(Y-OMe)RC-NH_2

no.	peptide[a]	platelet aggregation human PRP/ADP IC_{50} (μM)	IC_{50} (μM) $\alpha_{IIb}\beta_3$ ELISA	$\alpha_5\beta_1$ ELISA	$\alpha_v\beta_5$ ELISA
32	Ac-CNPRGD(Y-OMe)RC-NH_2	0.22	0.029	8.20	4.70
41	Ac-CNPRGD(Y-OMe)KC-NH_2	1.0	0.038	1.56	5.82
42	Ac-CNPRGD(Y-OMe)EC-NH_2	3.4	0.020	6.24	9.47
43	Ac-CNPRGD(Y-OMe)AC-NH_2	4.5	0.050	0.84	8.85
44	Ac-CNPRGD(Y-OMe)(D-R)C-NH_2	7.3	0.220	>10.00	>10.00
45	Ac-CNPRGD(Y-OMe)OC-NH_2	4.9	0.050	0.44	7.7
46	Ac-CNPRGD(Y-OMe)(Cit)C-NH_2	7.3	0.352	0.44	3.46
47	Ac-CNPRGD(Y-OMe)LC-NH_2	7.6	0.248	>10.00	7.14

[a] Non-natural amino acid abbreviations are given in ref 6.

conformationally restricting Pmc and Pmp residues did not significantly alter the relative platelet aggregation potency and had no significant effect on the selectivity compared to analogue **32**. The replacement of Cys by Mpr also resulted in no significant change in potency, suggesting that there is little interaction of the N-terminal acetyl group with $\alpha_{IIb}\beta_3$.

Effects of Arg Substitutions at Position 4 on Analogue 32 (Table 7). Finally, we examined the effect of the substitution of Arg at position 4 with other amino acids. The substitution of Arg with neutral amino acids (Ala, Cit, and Leu) resulted in substantial decreases in platelet aggregation potency (analogues **43**, **45**, and **47**). Similarly, the substitution of Arg with an acidic amino acid (Glu) led to a 15-fold decrease in potency (analogue **42**) while substitution with Lys resulted in a compound that retained 20% of the inhibitory activity (analogue **41**). This suggests that there is a specific binding pocket for a basic functional group at this position. However, the introduction of D-Arg in position 4 also led to a less favorable interaction of the peptide with $\alpha_{IIb}\beta_3$, resulting in a decrease in potency (analogue **44**). Therefore, it is concluded that the Arg residue at position 4 interacts with the receptor in a very stereospecific manner.

This series of analogues demonstrated that the Arg residue at position 4 plays a very important role in the binding of these analogues to $\alpha_{IIb}\beta_3$. The structure–activity relationship studies suggest that the R-G-D-Ar-R (Ar = hydrophobic residue) sequence is the essential pharmacophore (Figure 1) that is responsible for their high $\alpha_{IIb}\beta_3$ binding affinity and very high selectivity.

More importantly, several peptides in Table 7 were administered to dogs at doses capable of inhibiting *ex vivo* platelet aggregation by >95%. The peptides possessing the arginine at position 4 were the only ones that did not prolong template bleeding time at these concentrations. The results of this study, to be published elsewhere, are summarized in Table 8.

It is clear that the positive charge of the Arg at position 4 not only contributes binding affinity to $\alpha_{IIb}\beta_3$ but also is the primary element that allows the separation of

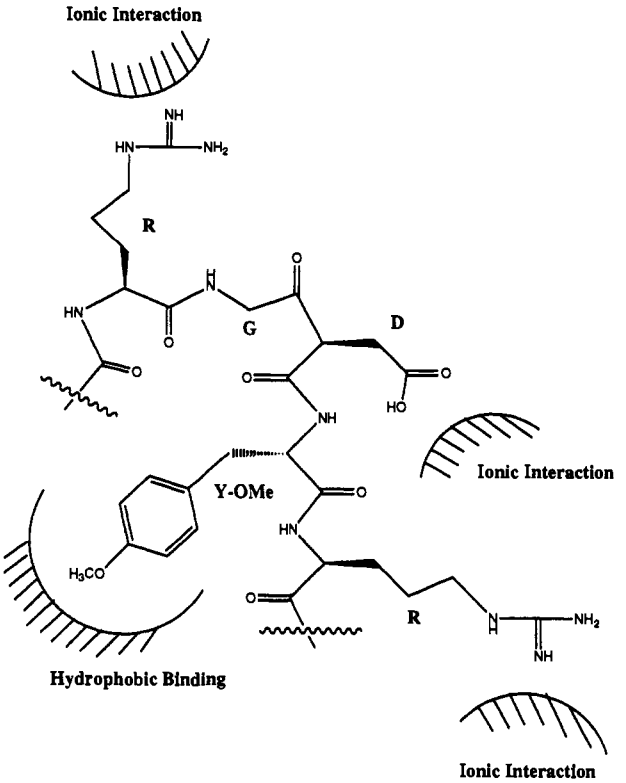

Figure 1. Schematic representation of R-G-D-Ar-R pharmacophore.

inhibitory activity toward platelet aggregation and the potential to control template bleeding. This holds true in another peptide, analogue **48**, which has an IC_{50} of 0.7 μM in the platelet aggregation assay. Although there is some controversy regarding the correlation between template bleeding measurements and bleeding events in a patient population,[60] the potential complications from surgical bleeding and blood loss should not be ignored. If this class of molecules can show clinical efficacy in humans without significantly affecting bleeding time, they may provide a strong margin of safety for use in patients with cardiovascular complications.

Table 8. Peptide Effect on Template Bleeding Time

no.	peptide	bleeding times[a,b] (min)
32	Ac-CNPRGD(Y-OMe)RC-NH_2	3.0 (5.5)[b]
42	Ac-CNPRGD(Y-OMe)EC-NH_2	>30
43	Ac-CNPRGD(Y-OMe)AC-NH_2	>30
47	Ac-CNPRGD(Y-OMe)LC-NH_2	21
48	Ac-CNPKGD(Y-OMe)RC-NH_2	3.5

[a] To measure bleeding time, two small cuts were made in the forearms of animals with a template bleeding time device (Simplate-II, Organon Technika, Durham, NC), and the exuded blood was absorbed onto Whatman no. 2 filter paper. The template bleeding time was considered to be the time after cutting at which the absorption of blood was no longer apparent. Peptide 32 was tested using the tongue to measure template bleeding time with identical results (data not shown). [b] Concentrations of peptides are high enough to cause >95% inhibition of *ex vivo* platelet aggregation. The bleeding time indicated in the parentheses was measured at a 5-fold higher concentration of peptide. The agonist used for *ex vivo* measurement of platelet aggregation was 10 μM ADP. *Ex vivo* platelet aggregation using 10 μg/mL collagen as agonist was also inhibited by >95% in this experiment with peptide 32 (data not shown).

The ability to separate the activities of platelets has been extended to an *in vivo* model. Analogue 32, also known as TP9201, was found to accelerate thrombolysis and prevent reocclusion in a canine experimentally induced coronary artery thrombosis model without an effect on template bleeding.[61,62] Other hemodynamic parameters also remained unchanged. These characteristics have led to the clinical development of this peptide.

Experimental Section

Peptide Synthesis. Peptide syntheses were performed by a solid-phase method[50,51] utilizing an automated synthesizer (Applied Biosystems, Inc. Model 431A). Carboxamide peptides were synthesized with *p*-methylbenzhydrylamine (*p*MBHA) resin, and peptides with C-terminal acids were synthesized with chloromethylated resin. Amino-terminal *tert*-butyloxycarbonyl protection was employed for all amino acids. The side-chain protection was Arg(Tos), D-Arg(Tos), Asp(β-cHex), Cys(*p*MeBzl), Glu(OBzl), His(Tos), Lys(Cbz), Orn(Cbz), Pen(*p*MeBzl), D-Pen(*p*MeBzl), Ser(OBzl), Tyr(2-Cl-Cbz), Pmp(4-MeBzl)-OH.[6] Boc-Pmc(*p*MeBzl)-OH,[56] Boc-Tyr(Et)-OH, and Boc-Tyr(*n*-butyl)-OH[57] were synthesized in our laboratory. Dicyclohexylcarbodiimide and hydroxybenzyltriazole were used in the coupling reactions, which were monitored by the ninhydrin test.

For the preparation of peptides with N-terminal acetylation, the peptides were acetylated using a mixture of acetic anhydride (20 equiv) and diisopropylethylamine (20 equiv) in *N*-methylpyrrolidone.

The peptides were removed from resin and deprotected with anhydrous hydrogen fluoride (HF;10 mL/g of resin-bound peptide) containing anisole (1 mL/g) at 0 °C for 60 min. After the evaporation of HF, the residue was washed with anhydrous ether, and the crude peptides were extracted with water or 15% aqueous acetic acid. The aqueous fractions were combined and lyophilized.

The crude acyclic peptide was dissolved (0.5 mg/mL) in 0.1 M ammonium bicarbonate and stirred open to the air. The course of the reaction was monitored via HPLC. After cyclization was complete (several hours to several days), the solution was filtered and purified via preparative RP-HPLC on a C_{18} Delta-Pak, 15 μm, 300A, 47 × 300 mm, eluting with a linear acetonitrile gradient (0–40%) containing a constant concentration of trifluoroacetic acid (0.1%, v/v) over 20 min at a flow rate of 40 mL/min.

Peptide Analysis. The purified peptides were analyzed by analytical reversed-phase HPLC on C-18 columns (Vydac, 5 μm, 300A, 4.5 × 250 mm). The purified peptides, recovered by lyophilization of the HPLC fractions, were at least 98% pure. The solvent system used for analytical HPLC was a binary system, water containing 0.1% TFA and acetonitrile containing 0.1% TFA as the organic modifier, and the solvent programs involved linear gradients as follows: (1) 0% to 40% acetonitrile over 15 min with flow rate of 1.5 mL/min; (2) 0% to 50% acetonitrile over 15 min with flow rate of 1.5 mL/min; (3) 0% to 60% acetonitrile over 15 min with flow rate of 1.5 mL/min.

All peptides were characterized by FAB (fast atom bombardment) mass spectroscopy and amino acid analysis. FAB mass spectroscopy was performed at Mass Spectrometry Service Laboratory, Department of Chemistry, University of Minnesota. Amino acid analysis was performed on Pickering Labs-Trione amino acid analyzer, equipped with a Spectra-Physics UV detector. Hydrolysis of peptide samples for AAA was carried out in vapor phase on 1-mg samples with 6 N constant-boiling HCl (1 mL), which were degassed and sealed under vacuum and then heated for 24 h at 110 °C.

Receptor and Ligand Purifications. Receptors were purified according to published procedures[52] with some modifications. Briefly, vitronectin receptor ($\alpha_v\beta_5$) was purified by RGD peptide-affinity chromatography from 100 mM octyl glucoside (OG)-extracted human placenta. After extraction, the suspension was adsorbed to a Sepharose CL4B column and then applied to a GRGDSPK affinity column. Except where stated, all procedures were carried out at 4 °C. The peptide column was washed with five volumes of Tris-buffered saline (TBS) containing 3 mM Ca^{2+} and 50 mM OG and then with five column volumes of TBS containing 3 mM Ca^{2+} and 50 mM OG at room temperature. Elution of bound receptor was achieved at room temperature with TBS containing 10 mM EDTA and 50 mM OG. Finally, 12 mM Ca^{2+} was added to eluted fractions.

Fibronectin receptor ($\alpha_5\beta_1$) was similarly purified from 100 mM OG-extracted human placenta using a procedure identical to that for the $\alpha_v\beta_5$ up to and including the initial Sepharose chromatography step. However, following this step, the Sepharose CL4B column flow-through was brought to 3 mM Mn^{2+} and the resulting solution was run over a 110 kDa fibronectin fragment-affinity column. Washing and elution steps were the same as those used in purifying vitronectin receptor, with the exception of the use of $MnCl_2$ rather than $CaCl_2$ in the wash buffer.

Platelet glycoprotein $\alpha_{IIb}\beta_3$ was purified from outdated human platelets. Briefly, the platelets were centrifuged for 10 min at 800*g* to pellet RBC's. The platelets were then washed three times with 20 mM Tris-HCl, 150 mM NaCl (TBS), 1 mM EDTA, 0.2% glucose, pH 7.5, and centrifuged at 1500*g* to pellet cells. Cells were lysed in two pellet volumes of TBS, 100 mM OG, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0.1 mM PMSF, followed by centrifugation at 30000*g*. The supernatant fraction was collected and loaded onto a Sepharose 2B column, previously equilibrated in TBS, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0.1 mM PMSF, 100 mM OG. Flow-through from the Sepharose 2B column was collected and passed over a GRGDSPK-Sepharose affinity column. The column was eluted with TBS containing 50 mM OG and 1 mg/mL GRGDSP. The fractions, after collecting and pooling, were diafiltered and concentrated on an Amicon YM 30 filter.

Human fibrinogen is purchased from Calbiochem.

$\alpha_5\beta_1$ ELISA Assay. Peptide binding to purified $\alpha_5\beta_1$ was determined by using a competitive enzyme-linked immunosorbent assay (ELISA) in which fibronectin is immobilized and the binding of solubilized $\alpha_5\beta_1$, in the presence of various concentrations of a peptide analogue, is detected with a polyclonal anti-FnR antibody followed by a labelled anti-rabbit IgG conjugate.[52]

Microtiter plates were coated overnight at room temperature with 110 μL of human fibronectin (at 2 μg/mL) in TBS. The plates were washed three times with TBS that contained 0.05% Tween-20. $\alpha_5\beta_1$ receptor in TBS containing 20 mM octyl glucoside and 2 mM $MnCl_2$ was added to each well. Next, 50 μL of peptide in the same buffer was added in 10-fold serial dilutions. The plates were incubated for 3 h at room temperature and washed with 200 μL of the above TBS-Tween buffer. Bound receptor was detected by incubation with 100 μL of affinity-purified rabbit anti-human fibronectin receptor antibody for 2 h, washed twice with TBS-Tween and then distilled water. Affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (100 μL) was then added to each well and incubated overnight at room temperature. The following day, the plates were washed with TBS-Tween and then distilled water. Then, 100 μL of substrate mixture (10 mg of *o*-phenylenediamine in 25 mL of 0.1 M citrate-phosphate buffer, pH 5.0, plus 6 μL of 30% H_2O_2) was added to

the plates and allowed to develop in the dark. The development process was stopped by adding 50 μL of 4 N H_2SO_4 to each well.

$\alpha_{IIb}\beta_3$ ELISA. Peptide binding to purified $\alpha_{IIb}\beta_3$ was determined in a similar ELISA system. The steps were the same as described above except the microtiter plates were coated with human fibrinogen at 10 μg/mL diluted in TBS, purified $\alpha_{IIb}\beta_3$ was diluted in TBS with 20 mM octyl glucoside containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$, and rabbit anti-$\alpha_{IIb}\beta_3$ was used to detect bound receptor.

$\alpha_v\beta_5$ ELISA. Peptide binding to purified $\alpha_v\beta_5$ was determined in a similar ELISA. The steps were identical with the $\alpha_5\beta_1$ ELISA except that microtiter plates were coated with purified human vitronectin diluted to 10 mg/mL in 0.1 M carbonate buffer (pH 9.6), purified human $\alpha_v\beta_5$ was diluted in TBS containing 20 mM octyl glucoside, 2 mM $MgCl_2$, and 2 mM $CaCl_2$, and affinity purified rabbit anti-VnR was used to detect bound receptor.

Platelet Aggregation Assay. *Ex vivo* platelet aggregation was determined by established spectrophotometric methods with a four-channel aggregometer (BioData-PAP-4, BioData Corporation, Hatboro, PA) by recording the increase in light transmission through a stirred suspension of PRP maintained at 37 °C. Aggregation was induced with ADP (10 μM) or collagen (10 μg/mL). Values were expressed as a percentage of aggregation. This represents the percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission, respectively.

Blood (20 mL) was withdrawn from the cephalic vein into a plastic syringe containing 3.2% sodium citrate. The platelet count was determined with a Haema Count MK-4/HC system (J. T. Baker, Allentown, PA). Platelet-rich plasma (PRP), the supernatant present after centrifuging anticoagulated whole blood at 1000 rpm for 5 min (140*g*), was diluted with platelet-poor plasma PPP) to achieve a platelet count of 200 000/μL. PPP was prepared after the PRP was removed by centrifuging the remaining blood at 12,000*g* for 10 min and discarding the bottom cellular layer.

All aggregation studies were performed at 37 °C with a constantly stirred suspension of 2×10^8 platelets/mL. Peptides and stimulants were added to these suspensions in 1% dilutions. The PRP and gel-filtered platelets were used within 3 h from the time of blood collection.

Peptide anti-aggregation potencies were determined from dose–response curves for the inhibition of the maximum aggregation responses stimulated by physiologic doses of ADP (10 μM) and thrombin (2 U/mL). The 50% inhibitory concentration of each peptide (IC_{50}) was determined by regression analysis of these curves. Individual data points are accurate to ±15%.

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Supplementary Material Available: One table giving amino acid analyses, FAB mass spectral data, HPLC retention times and TLC R_f values (5 pages). Ordering information is given on any current masthead page.

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X_1	X_2	X_3	X_4	R	G	D	X_5	X_6	X_7	X_8
-4	-3	-2	-1	0	1	2	3	4	5	6

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