Non-Peptide Fibrinogen Receptor Antagonists. 1. Discovery and Design of Exosite Inhibitors[1]

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When a blood vessel is injured, either by the acute damage of wounding or clinical interventions, or more chronically by the pathophysiological processes of atherosclerosis, platelets are activated to adhere to the disrupted surface and to each other. This activation, adherence, and aggregation may lead to occlusive thrombus formation in the lumen of the blood vessel.[2,3] Platelet activation and aggregation are also implicated in acute myocardial infarction, in reocclusion following thrombolytic therapy and angioplasty, in transient ischemic attacks, and in a variety of other vaso-occlusive disorders.[4-6]

The final obligatory step in platelet aggregation is the binding of fibrinogen to an activated membrane-bound glycoprotein complex, GP IIb/IIIa.[7-11] During this activation process, GP IIb/IIIa undergoes an ill-defined change in the spatial orientation of extracellular domains resulting in the exposure of binding sites for fibrinogen.[12-15] Since the binding of fibrinogen to its platelet receptor is an obligatory component of normal aggregation, inhibition of this GP IIb/IIIa-mediated event constitutes an attractive antithrombotic mechanism.

There are six putative recognition sites within fibrinogen for GP IIb/IIIa and thus fibrinogen acts as a hexavalent ligand to crosslink GP IIb/IIIa sites on adjacent platelets.[16] Attempts have been made to use fibrinogen fragments and synthetic peptides to define the sequence within fibrinogen which mediates the GP IIb/IIIa–fibrinogen interaction and thus aggregation. The tripeptide sequence arginyl-glycyl-aspartyl (RGD) is believed to represent the minimal sequence necessary for binding to GP IIb/IIIa.[17] RGD occurs four times in fibrinogen twice in each of the two Aα chains, thus contributing four of the six putative recognition sites within fibrinogen. The other two sites occur in the C-terminal 12 amino acid segments of the two γ chains.

Although the tripeptide RGD-amide is sufficient to inhibit platelet aggregation at high concentractions, the most potent inhibitors of the interaction between fibrinogen and GP IIb/IIIa are antibodies directed against IIb/IIIa, the disintegrin family of RGD-containing proteins, and cyclic RGD-containing peptides.[18-25] We now report the discovery and development of novel, low molecular weight, non-peptide fibrinogen receptor antagonists that

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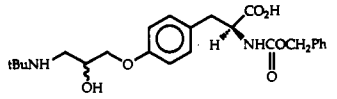
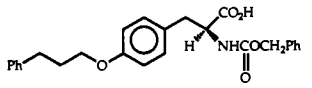
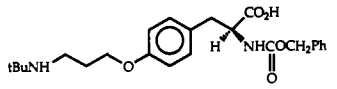
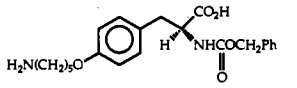
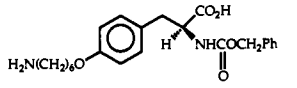
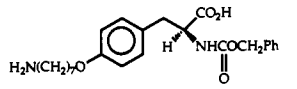
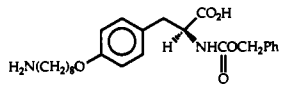
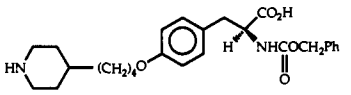
Table I. Modifications of 1 at the N-Terminus

compd	structure	$IC_{50}(\mu M)^{a,22}$
	Arg-Gly-Asp-Ser	26
1		27
2		>1000
3		22
4		36
5		26
6		2.6
7		40
8		0.26

[a] Platelet aggregation with human gel filtered platelets (GFP) was measured by the light transmittance method at 37 °C with 2×10^8 platelets/mL, 0.1 mg/mL human fibrinogen, and 1 mM $CaCl_2$. Aggregation was initiated by adding 10 μM ADP after all other components were added. The rate of aggregation in the absence of inhibitor served as the control and values reported are the concentration necessary to inhibit the rate of aggregation by 50%. At least two determinations are made for each compound.

mimic the Arg-Gly-Asp sequence of active peptides and incorporate an additional, potency-enhancing interaction.

A directed search of the Merck Sample Collection for compounds that possessed amino and carboxylate functionalities that were separated by through-bond distances of 10–20 Å (approximating the distance between the basic guanidine and the acidic carboxylate of Arg-Gly-Asp) provided the lead compound 1, an analog of L-tyrosine wherein the phenolic oxygen was alkylated with a β-blocker side chain. This compound displayed an $IC_{50} = 27$ μM for inhibition of ADP-mediated platelet aggregation and a $K_i = 25$ μM for inhibition of ^{125}I-fibrinogen binding,[26] suggesting that 1 was a specific inhibitor of GP IIb/IIIa-mediated platelet aggregation.

Synthetic modification of 1, which was of comparable potency to Arg-Gly-Asp-Ser, centered initially on optimizing the N-terminus features (Table I). The presence of a basic functionality at this site was required for potency, as would be expected of an Arg-Gly-Asp mimic, as shown by the lack of activity of 2, where phenyl replaces *tert*-butylamino, and the *N*-Boc analog of 1 (data not shown). Removal of the secondary hydroxyl group of 1 gave the structurally simplified analog 3, with no loss in potency.

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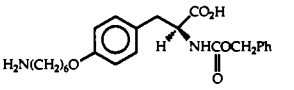
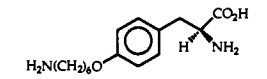
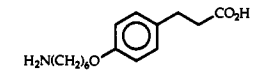
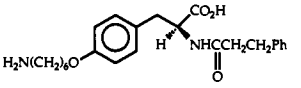
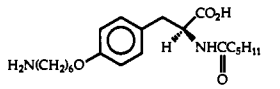
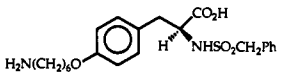
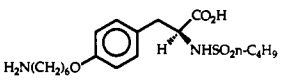
Table II. Modifications of 5 at the C-Terminus

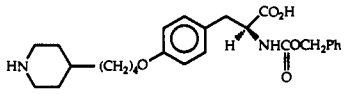
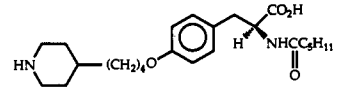
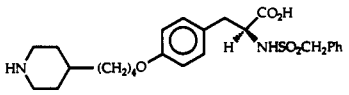
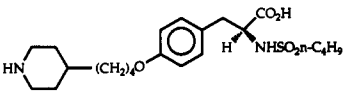
compd	structure	$IC_{50}(\mu M)$
5		26
9		35
10		>100
11		8
12		3.8
13		2.0
14		0.78

Next, the distance between the tyrosine ring and the terminal basic center was altered by varying the number of methylene units in the connecting chain. Thus, while 4, 5, and 7 displayed IC_{50} values in the 26–40 μM range, compound 6, possessing a seven methylene unit linkage, was found to be approximately 10-fold more potent. Finally, conformational restriction in the amino terminal chain by bridging across the final four atoms of 6 afforded the piperidine 8, which displayed an $IC_{50} = 0.26$ μM for inhibition of ADP-mediated platelet aggregation. Overall, therefore, optimization of chain length at the amino terminus in conjunction with appropriate conformational restraints provided a 100-fold increase in potency (8 vs 1) with removal of a chiral center and only modest increase in molecular weight.

A similar effort at structural optimization was directed at the C-terminus of the aminohexyl derivative 5 (Table II). Removal of the carbobenzyloxy group of 5 gave the free amino acid 9 with no loss in potency. However, elimination of the polar α-amino substituent provided the phenylpropionic acid 10, which was >3-fold less potent than 9. The critical replacement of the carbamate oxygen of 5 with methylene gave the *N*-phenylpropanoyl derivative 11, which was 3-fold more potent than 5, and a further 2-fold increase in potency was achieved with 12, wherein the aliphatic hexanoyl moiety replaced phenylpropanoyl. The structural origin of the 6-fold potency difference between 5 and 12 is a matter of ongoing study; however, this increment may reflect features of enhanced hydrogen bonding or electrostatic interaction at the amide carbonyl and nitrogen sites.

With this aspect of design as a guide, the key observation was made that, for this series of tyrosine-derived fibrinogen receptor antagonists, potency was enhanced beyond that of carbamates and amides by the introduction of sulfonyl substituents on the α-amino group. Thus, replacement of the carbobenzyloxy group of 5 with benzylsulfonyl provided sulfonamide 13 ($IC_{50} = 2$ μM), while replacement with *n*-butylsulfonyl afforded 14 ($IC_{50} = 0.78$ μM). The

Table III. Modifications of 1 at N- and C-Terminii

compd	structure	$IC_{50}(\mu M)$
8		0.26
15		0.035
16		0.018
17		0.011

3-fold potency increment between 13 and 14 suggests the presence of a hydrophobic interaction at the α-amino substituent site, in addition to the ionic component previously discussed. Overall, this sequence of structural optimization at the C-terminus resulted in the replacement of the carbobenzyloxy group of 5 with *n*-butylsulfonyl, attendant with a 30-fold potency enhancement and a decrease in molecular weight.

The extent of potency enhancement in molecules that possessed both optimized N- and C-terminus functionality was of considerable interest (Table III). When the piperidine analog 8 (IC_{50} = 0.26 μM) was functionalized at the C-terminus with hexanoyl to provide 15 (IC_{50} = 0.035 μM), a 7-fold increase in potency was observed. Gratifyingly, this was comparable to the potency increment between 5 and 12. Similarly, replacement of the hexanoyl group of 15 with benzylsulfonyl provided 16 (IC_{50} = 0.018 μM), which displayed a 2-fold increase in potency over 15. Finally, utilization of the *n*-butylsulfonyl group provided 17 (L-700,462) (IC_{50} = 0.011 μM), whose 24-fold potency increase over 8 was comparable to the increase in potency observed when 14 is compared to 5. In line with the platelet aggregation potency, 17 displayed an IC_{50} = 5 nM for inhibition of ^{125}I-fibrinogen binding.

The specificity of 17 for inhibition toward a variety of integrins, a key feature of potential antithrombotic utility, was appraised by measuring the IC_{50} for inhibition of human umbilical vein endothelial cell (HUVEC) attachment to fibrinogen (Fg), vitronectin (Vn), and fibronectin (Fn).[27] As shown in Table IV, approximately 10000-fold higher concentrations of 17 were required to inhibit these HUVEC adhesion events than was required to inhibit platelet aggregation, demonstrating selective inhibition by 17 of the platelet GP IIb/IIIa receptor. This level of integrin selectivity for 17 is clearly enhanced over that of the snake venom peptide Echistatin and is comparable to that found for peptide inhibitors[24] that employ replacement of arginine with amines.

A consideration of molecular models suggests that 17 functions as a mimic of Arg-Gly-Asp wherein the piperidine N replaces the basic guanidino moiety of Arg and the tyrosine carboxyl group acts as an aspartic acid carboxyl surrogate. There is clear irony in the result that the search for non-peptide antagonists of the platelet fibrinogen receptor has led to the discovery of an amino acid, L-tyrosine, which can efficiently replace the Gly-Asp dipeptide core of Arg-Gly-Asp inhibitors. This observation is intriguing in view of the considerable potency decreases and selectivity changes that attend peptide synthetic variation at the arginine, glycine, and aspartic acid sites.[21,25,28-31]

Table IV. Inhibition of HUVEC Attachment to Fibrinogen, Vitronectin, and Fibronectin[a,27]

compound	$IC_{50}(\mu M)$ (platelet)	$IC_{50}(\mu M)$ (HUVEC) Fg	Vn	Fn
Echistatin	0.033	0.00045	0.00072	0.00078
17 (L-700,462)	0.011	260	100	>300

[a] Human umbilical vein endothelial cells (HUVEC) were used between passages 2 and 7. Following removal from monolayer, cells were allowed to attach to microtiter plates containing human fibrinogen (Fg), human vitronectin (Vn), or human fibronectin (Fn). After 75 min at 37 °C, unattached cells were removed by gentle washing and adherent cells were quantitated by measuring glucosaminidase activity. Binding of HUVEC to fibrinogen was blocked by specific monoclonal antibodies to α_V (AMAC) or $\alpha_V\beta_3$(23C6) but not α_5 or α_2, to vitronectin by antibodies directed against $\alpha_V\beta_3$ (AMAC) but not α_5 or α_2, and to fibronectin by antibodies directed against α_5 (Telios) but not α_V or $\alpha_V\beta_3$. Thus, HUVEC attachment to these three substrates monitors $\alpha_V\beta_3$ and $\alpha_5\beta_1$ activity.

Molecular modeling further suggests that the potency enhancing (*S*)-$NHSO_2n$-C_4H_9 group of 17 experiences favorable noncovalent interaction at a site on GP IIB/IIIa which has not previously been utilized in the same manner by either linear or cyclic Arg-Gly-Asp based fibrinogen receptor antagonists. Thus, we have termed this interaction as occurring at an "exosite".[31] It is anticipated that GP IIb/IIIa inhibitors that exploit exosite potentiation will offer opportunities for structural design that previously have not been available.

In summary, we have reported the discovery of O-alkylated L-tyrosine analogs as Arg-Gly-Asp mimics that function as inhibitors of platelet aggregation. Structural optimization at the N- and C-termini of lead compound 1 provided the potent antagonist 17 (L-700,462) (IC_{50} = 0.011 μM), a novel, low molecular weight, selective GP IIb/IIIa antagonist. This inhibitor appears to derive significant potency enhancement from interaction at a previously unrecognized exosite on GP IIb/IIIa. Further structure/activity, molecular modeling, and biological investigation is underway and will be reported in due course.

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