Design and Synthesis of RGD Mimetics as Potent Inhibitors of Platelet Aggregation

ELIZABETH POLLINA

Vascular diseases, such as heart attacks and strokes, are known to be caused by the aggregation of platelets which form clots within blood vessels. These diseases can be prevented through the use of drugs that inhibit platelet aggregation by blocking a key platelet receptor, GPIIb/IIIa. The recognition of the arginine-glycine-aspartic acid (RGD) amino acid sequence by this receptor has been exploited in formulating a drug strategy to prevent vascular diseases. Thus, the RGD sequence or its mimetics have been incorporated into drug candidates that inhibit platelet aggregation. Through a process of rational design, mimetics of the RGD sequence were synthesized, providing deeper insight into the structural requirements for high inhibitory activity. These mimetics were found to be extremely potent inhibitors of platelet aggregation.

Introduction

Hemostasis and Thrombosis. The natural process of hemostasis is the maintenance of a delicate equilibrium that permits the unobstructed flow of blood in vessels, while also allowing its coagulation as a spontaneous reaction to an injury. This equilibrium exists as a dynamic exchange between various clotting and non-clotting factors. Platelets play a crucial role in the maintenance of hemostasis. However, a malfunction of this process can lead to a loss of hemostatic equilibrium within the blood vessel resulting in the formation of an internal clot, a hemostatic plug.

Platelet physiology is regulated by a variety of receptors on the platelet surface. These receptors participate in the process of thrombosis, i.e., platelet-mediated formation of a hemostatic plug. There are four fundamental steps involved in thrombosis (Figure 1): 1) activation of the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) receptor complex by means of different agonists, such as certain blood proteins and enzymes; 2) adhesion of a platelet to vascular lesions or plaque by means of cell surface receptors; 3) platelet aggregation into a large mass collectively known as a thrombus; 4) coagulation, which occurs as a cascade of several biochemical processes following platelet aggregation that culminate in the creation of an impervious three-dimensional meshwork.[1]

The key to abnormal thrombotic events is the lack of a mechanism in platelets to distinguish between a blood vessel rupture in need of repair and a rough surface on the wall of an otherwise undamaged blood vessel. Inside the blood vessels, dead tissue or deposits of cholesterol may form sharp or rough surfaces on the vessel wall. Passing platelets in the bloodstream may attach to these surfaces and aggregate. This process of aggregation is caused by the cross-linking of activated GPIIb/IIIa receptors on different platelets by certain blood glycoproteins, mainly fibrinogen.[2] The resultant thrombus may lead to an interruption in the normal flow of blood. Restriction of blood flow eventually leads to thrombotic disorders including heart attacks and strokes.

Heart attacks and other thrombotic disorders are among the major causes of death that plague modern society with increasing incidence. It is therefore worthwhile to search for drugs to treat or prevent thrombosis. Several antithrombotic therapies can be described which can target different stages of the coagulation cascade. However, the GPIIb/IIIa-fibrinogen association is an essential and specific step in thrombosis, and is currently viewed as an attractive target for drug design efforts. In this regard, the development of GPIIb/IIIa receptor antagonists may be a useful strategy for the treatment and prevention of various cardiovascular and cerebrovascular diseases.

GPIIb/IIIa Antagonists and the RGD Sequence. The glycoprotein IIb/IIIa (GPIIb/IIIa) receptor on platelets plays a crucial role in the process of thrombosis. GPIIb/IIIa belongs to a superfamily of cell-surface receptors known as integrins that can recognize molecules carrying the Arg-Gly-Asp (RGD) binding sequence in different conformations.[3] Inhibition of platelet aggregation and avoidance of all the subsequent steps that lead to a thrombotic episode can be achieved by preventing the binding of fibrinogen to GPIIb/IIIa. A logical way to inhibit this binding is by creating a receptor antagonist that can competitively block the fibrinogen binding site on GPIIb/IIIa (Figure 1). The inability of this antagonist, unlike fibrinogen, to cross-link platelets would effectively inhibit platelet aggregation. The presence of the RGD binding sequence in fibrinogen and related glycoprotein ligands has led many research groups to focus on the

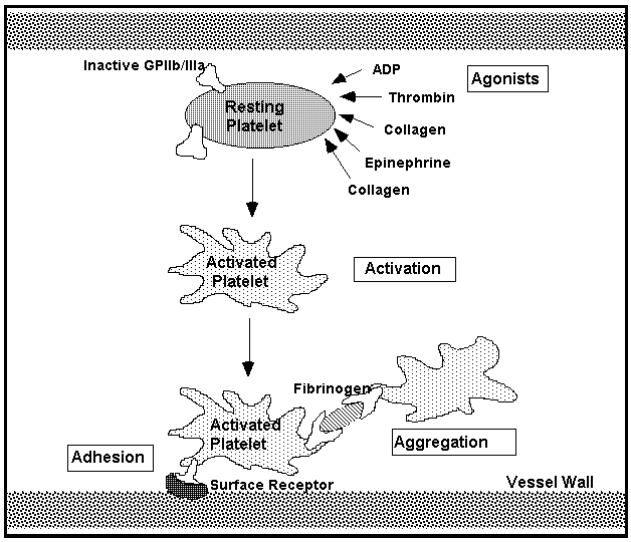
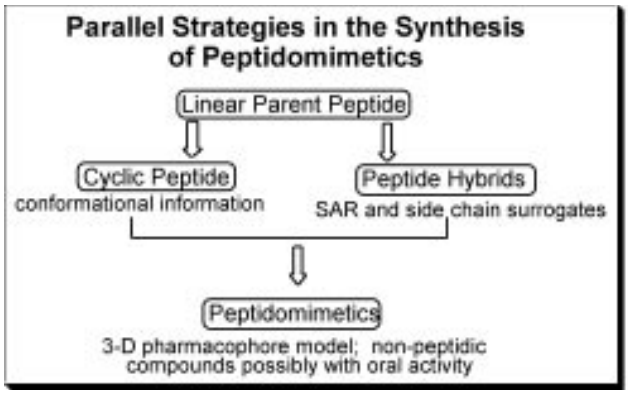
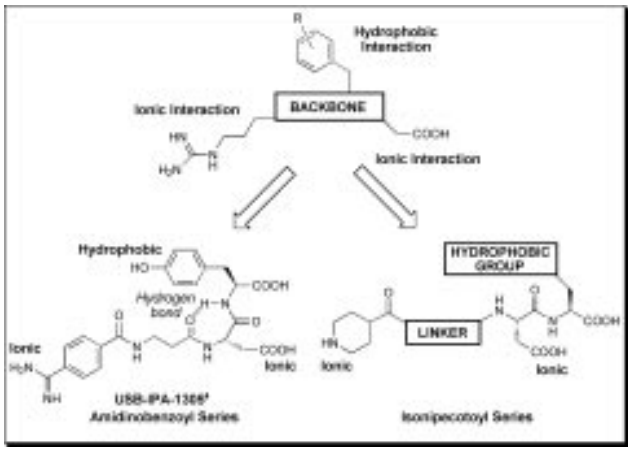


Figure 1. Process of thrombosis.

ELIZABETH POLLINA is a first-year student attending Dartmouth College. She completed the research described in this article under the supervision of Dr. Iwao Ojima, with the assistance of Dr. Subrata Chakravarty, at the State University of New York at Stony Brook. Ms. Pollina was honored as a Westinghouse Science Talent Search finalist for this work, and she was awarded fourth place in the DURACELL/NSTA Scholarship Competition.



Scheme 1.



Scheme 2. The three-point pharmacophore model for GPIIb/IIIa antagonists and its representation by a potent antithrombotic agent, USB-IPA-1305.[4]

development of antithrombotic agents by designing molecules either containing or mimicking the RGD sequence. The aim of this project is to develop mimics of the RGD sequence by rational design.

Strategy of Rational Drug Design: Peptides to Peptidomimetics. Current efforts in drug development are aimed at developing non-peptidic inhibitors that mimic the structure and function of bioactive peptides. Known as peptidomimetics, these compounds have many advantages over their parent peptides.[4] Peptides are characterized by their rapid digestion by proteolytic enzymes, low absorption through intestinal walls, and potential immunogenic reactions.[5] The proper design of peptidomimetics seeks to avoid these peptide related complications. Peptidomimetic design also involves the discovery of the bioactive conformation of the parent peptide and its successful translation into a conformationally constrained molecule. This is extremely relevant because peptides, although inherently flexible in nature, are believed to adopt only one biologically active conformation at the site of interaction. The loss of rotational freedom in the constrained peptidomimetic should therefore greatly enhance its activity when compared to that of the peptide.

There are several strategies for the design of peptidomimetics. In order to obtain a peptidomimetic, a combination of different strategies is often found in scientific literature. One strategy is to optimize the parent peptide, or lead, by synthesizing peptide hybrids (Scheme 1). A lead peptide may be modified by altering important functionalities in individual amino acid residues as well as the backbone components, such as amide bonds, with chemical units that mimic their structure and function. The use of conformationally constrained units often supplies important information about bioactive conformations. Through structure-activity relationships (SAR) of these rationally designed peptide hybrids, important information is collected leading to a pharmacophore model, containing relevant structural information required for optimal biological activity. This model can serve as a starting point for the design of novel peptidomimetics.

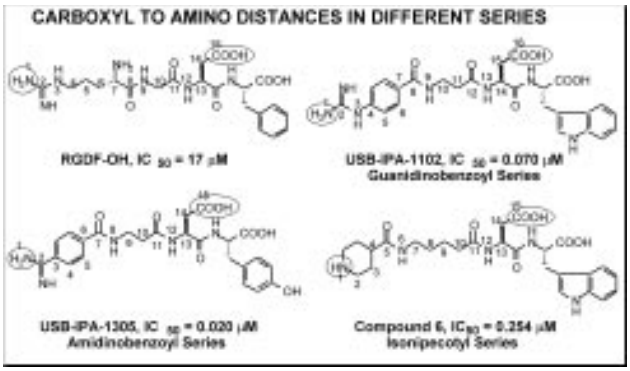
Another strategy in developing peptidomimetics is to cyclize a linear peptide into a cyclic peptide. A cyclic peptide has reduced conformational freedom compared to its linear predecessor and provides an excellent opportunity to define a bioactive conformation.[6] This cyclic peptide strategy may lead to a proposal for an idealized three-dimensional pharmacophore model. In both the peptide hybrid and cyclic peptide approaches, three-dimensional molecular modeling and nuclear magnetic resonance (NMR) spectroscopy can be used as time- and effort-saving tools for the analysis of pharmacophore models.

Basis of Project. The rational design of the peptide hybrids described in this article are based on a three-point pharmacophoric binding model (Scheme 2). The three points of interaction in the idealized inhibitor are hypothesized to interact at two ionic (one cationic and one anionic) and one hydrophobic binding sites present on GPIIb/IIIa.[7] The distances and spatial orientation of these three points are critical for enhanced biological activity. The two ionic components are believed to be separated by a slightly curved backbone. The hydrophobic component is accommodated on the convex side of the backbone. This orientation of the hydrophobic group in the lead peptide hybrid, USB-IPA-1305,[4] is stabilized by a hydrogen bond to obtain a γ-turn, a structural feature commonly encountered in biomolecules.

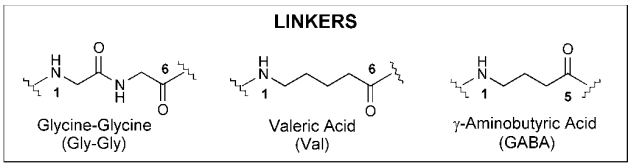
Starting from the pharmacophore model represented in Scheme 2, the following modifications were planned.

1. Introduction of isonipecotyl group as an arginine side chain mimetic. To optimize activity, the guanidinobenzoyl and amidinobenzoyl groups have been used as conformationally constrained substitutes for the arginine side chain (Scheme 3).[4] My proposal included the incorporation of the isonipecotyl group as a surrogate for the arginine side chain into the overall mimetic model. As depicted in Scheme 3, the nitrogen atom of the isonipecotyl groups is expected to serve the same function as the circled nitrogen atoms in the arginine side chain, amidinobenzoyl and guanidino groups. However, the nature of the nitrogen atoms is quite different in terms of both electronic and steric characteristics.

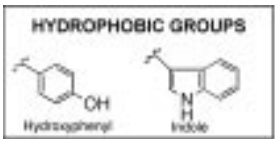
2. Variation of linker length. With the substitution of the amidinobenzoyl group with the isonipecotyl group, the linker length between the isonipecotyl and aspartyl residues must be adjusted for optimum potency (Scheme 4). Based on results of the amidinobenzoyl series, the ideal linker length was expected to be 6 atoms.[7] Thus, only three different linkers were used: valeric acid, a glycine-glycine combination, and γ-aminobutyric acid (GABA). GABA, a 5-atom linker was used as a negative test.



Scheme 3.



Scheme 4.



Scheme 5.

3. Use of different C-terminal hydrophobic groups. The nature of the C-terminal hydrophobic group may affect activity. Two hydrophobic amino acids were chosen for this study: tryptophan, containing the bulky indole ring, and tyrosine, containing the less bulky hydroxyphenyl ring (Scheme 5).

4. COOH to $CONH_2$. Parallel to the work on the isonipecotyl containing molecules, some modifications to the amidinobenzoyl series were also planned. Specifically, the replacement of the carboxyl with an amide terminus in active amidinobenzoyl containing leads was planned to better understand the contribution, if any, of the terminal carboxyl group in these inhibitors.

5. *Testing the γ-turn hypothesis.* The γ-turn hypothesis was tested by altering the point of attachment at the backbone of the hydrophobic residue, without apparently changing its spatial orientation with respect to the two ionic sites (Scheme 6). The molecules were further simplified by using β-alanine as an aspartic acid mimic devoid of the α-carboxylic acid group.

Hypothesis. The goal is to rationally design and synthesize peptide hybrids that efficiently inhibit platelet aggregation. Beginning with a lead peptide hybrid (USB-IPA-1305),[4] the following modifications are proposed (Scheme 7):

(1) Replace the amidinobenzoyl group with an isonipecotyl group and observe the effects of the substitution.

(2) Use different linkers of varying length to optimize the distance between the isonipecotyl and nitrogen and aspartyl side chain carboxyl group.

(3) Use different C-terminal amino acids of hydrophobic nature.

(4) Replace the carboxyl terminus in compounds of the amidinobenzoyl series with an amide.

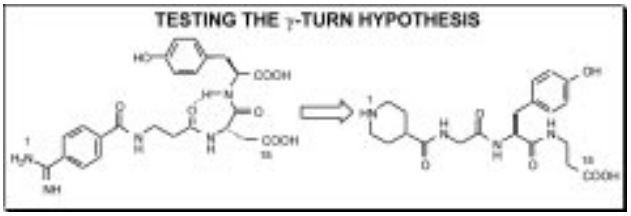
(5) Test the γ-turn hypothesis by altering the point of attachment of the hydrophobic group.

Based on the rationalization of the results obtained from these modifications, the contribution of these results to the overall pharmacophoric model will be assessed.

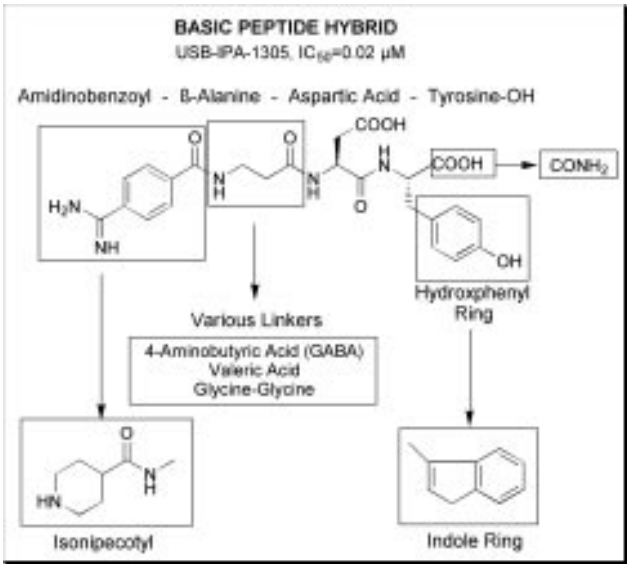
Methods

Materials. Solid phase synthesis on Wang resin and Rink amide MBHA resin was performed on the DuPont RamPS™ system. The amino acids and resins were purchased from Novabiochem, Inc. or Bachem Bioscience, Inc. All other chemicals were purchased from the Aldrich Chemical Company.

Solid Phase Peptide Synthesis (SPPS). *Preparation of resin and deprotection.* 9-Fluorenylmethoxycarbonyl (Fmoc)-amino acid-Wang resin or Rink amide MBHA resin (0.1 mmol) was warmed to room temperature. The resin was



Scheme 6.



Scheme 7.

washed three times with N,N-dimethylformamide (DMF). The Fmoc protecting group was deprotected by shaking the resin in 50% piperidine/DMF (3 mL) for 9 min. The resin was successively washed three times each with DMF, methanol (MeOH), and again DMF.

Synthesis of HOBt ester. Suitable side-chain protected Fmoc-amino acid (0.25 mmol) was suspended and stirred in dichloromethane (1.0 mL) at 0°C for 5 min. A 0.5 M solution of 1-hydroxybenzotriazole (HOBt)/DMF (0.5 mL of 0.5 M solution) was added to the suspension and stirred at 0°C for 5 min. To this suspension, 1,3-diisopropylcarbodiimide (DIC) (39 µL) was added and the resultant solution was stirred for 2 min. at 0°C followed by stirring for 5 min. at room temperature. This solution was then added to the cartridge containing the deprotected resin.

Coupling. Following the addition of the HOBt ester solution to the resin, the cartridge was shaken for 2 hr. at room temperature. The mixture was drained and the resin washed as described in A.

Kaiser Test. The Kaiser Test was performed to monitor the completeness of coupling of successive amino acids. A sample of the resin was added to a labeled 12x75 mm glass test tube along with 40 µL solution A (0.5 M ninhydrin in ethanol), 50 µL solution B (400% phenol in ethanol), and 100 µL solution C (potassium cyanide/pyridine). The contents were incubated at 100°C for 5 min. If the resin beads or solution were colored, the coupling was considered incomplete and was repeated until the beads and solution were colorless on this test.

Completion of the hybrid. The cycle was repeated from the deprotection step until the desired peptide sequence was obtained.

Cleavage of Completed Peptide from Resin. In the case of Wang resin-based peptides, the peptide resin was treated with trifluoroacetic acid (TFA) (3.8 mL), thioanisole (0.3 mL), ethanedithiol (EDT) (0.1 mL), phenol (50 µL), and trimethylsilylbromide (TMSBr) (0.675 mL) at 0°C for 1 hr. In the case of the Rink amide MBHA resin, the peptide resin was treated with TFA (5.8 mL), thioanisole (0.15 mL), ethanedithiol (0.1 mL), and phenol (0.05 mL) for 4 hr. at room temperature.

The resin was removed by filtering through a plug of glass wool and excess TFA and TMSBr were removed with a stream of nitrogen gas. The residue was treated with ether (25 mL) whereupon the product precipitated out. Three cycles of centrifugation and washing with ether yielded the crude product that was dried under a stream of nitrogen gas. All products were stored in a freezer at -20°C.

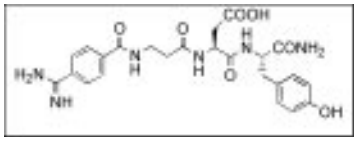
Analysis and Purification. *High Pressure Liquid Chromatography (HPLC).*[8] Both analysis and purification of products were carried out on a Waters 600E Multisolvent Delivery System equipped with a Waters 484 Tunable Absorbance Detectors using a µBondpak C18 reverse phase columns (analytical, 3.9 mm by 300 mm; preparative 19 m by 150 mm). Digitization of the data was performed on an NEC Powermate personal computer using Microsoft Baseline 810 software. The eluant system consisted of solution A (0.1% TFA in water) and solution B (0.1% TFA in 60% acetonitrile-40% water). Absorbance was measured at 254 nm.

Fast Atom Bombardment-Mass Spectrometer (FAB-MS). FAB-MS was used to verify the molecular weight of the synthesized peptides. The spectra were obtained on a KRATOS MS 80 system. These measurements were carried out at the University of California at Riverside mass spectral facility.

Bioassays. In vitro *platelet aggregation inhibitory assay (IC_{50}).*[8] Platelet aggregation was observed using a Lumi Aggregometer 400 VS. Adjusted platelet rich plasma (PRP) (0.45 mL) was taken in a glass cuvette and incubated in the aggregometer for 2 min. at 37°C while stirring at 1200 rpm. Peptide stock solution (36 µL) was added, followed by a 100 µM adenosine diphosphate (ADP) (54 µL) in TRIS buffer after a 1 min. delay. The transmission of light was compared against the standard containing platelet poor plasma (PPP) (0.45 mL) and then recorded graphically by the instrument. The experiment was repeated for lower concentration until at least three readings with appreciable positive slopes were obtained. The slopes were plotted against the logarithm of the concentration using Cricket Graph 5.0. The concentration at which the test compound showed 50% inhibition (measured as half the slope for the aggregation experiment of a control run using only TRIS buffer) was taken as the IC_{50}. The obtained IC_{50} was corrected against the standard peptide RGDF-OH (17µM).

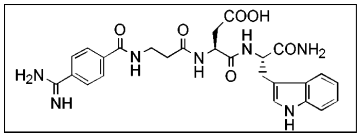
Peptide Hybrids.

Peptide Hybrid 1: AmBz-β-Ala-Asp-Tyr-NH_2



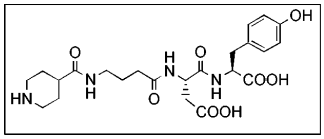
AmBz-β-Ala-Asp-Tyr-NH_2 was synthesized using standard DuPont RamPS™ method starting from Rink Amide MBHA resin. A white product (15 mg, 24% yield) was obtained after HPLC purification (99% purity, retention time 7.54 min. by analytical HPLC, 100%A-30 min.-90%B).

Peptide Hybrid 2: AmBz-β-Ala-Asp-Trp-NH_2



AmBz-β-Ala-Asp-Trp-NH_2 was synthesized using standard DuPont RamPS™ procedure starting from Rink Amide MBHA resin. A white product (9 mg, 16% yield) was obtained after HPLC purification (98% purity, retention time 14.45 min. by analytical HPLC, 100%A-30 min.-90%B).

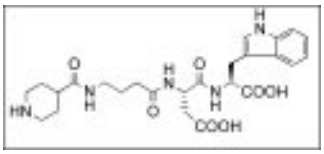
Peptide Hybrid 3: Isonip-GABA-Asp-Tyr-OH



Isonip-GABA-Asp-Tyr-OH was synthesized using standard DuPont RamPS™ procedure starting from Fmoc-Wang resin. A white product (8.4 mg, 17% yield) was obtained

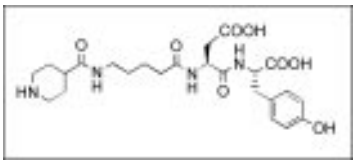
after HPLC purification (>99% purity, retention time 4.69 min., by analytical HPLC, 90%A-30 min.-100%B).

Peptide Hybrid 4: Isonip-GABA-Asp-Trp-OH



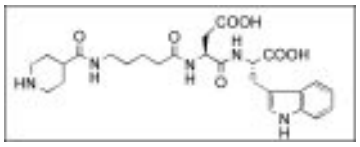
The Isonip-GABA-Asp-Trp-OH was synthesized using standard DuPont RamPS™ procedure starting from Fmoc-Wang resin. A white product (13.9 mg, 27% yield) was obtained after HPLC purification (92% purity, retention time 6.27 min., by analytical HPLC, 90%A-30 min.-100%B).

Peptide Hybrid 5: Isonip-Val-Asp-Tyr-OH



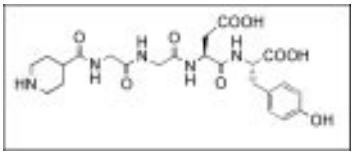
The Isonip-Val-Asp-Tyr-OH was synthesized using standard DuPont RamPS™ procedure starting from Fmoc-Wang resin. A white product (13.2 mg, 26% yield) was obtained after HPLC purification (96% purity, retention time 4.93 min., by analytical HPLC, 90%A-30 min.-100%B).

Peptide Hybrid 6: Isonip-Val-Asp-Trp-OH



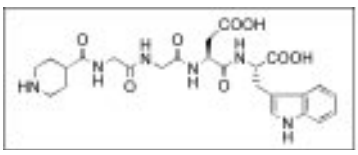
The Isonip-Val-Asp-Trp-OH was synthesized using standard DuPont RamPS™ procedure starting from Fmoc-Wang resin. A white product (18.5 mg, 35% yield) was obtained after HPLC purification (94% purity, retention time 6.38 min., by analytical HPLC, 90%A-30 min.-100%B).

Peptide Hybrid 7: Isonip-Gly-Gly-Asp-Tyr-OH



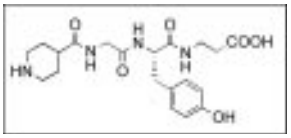
The Isonip-Gly-Gly-Asp-Tyr-OH was synthesized using standard DuPont RamPS™ procedure starting from Fmoc-Wang resin. A white product (36.0 mg, 69% yield) was obtained after HPLC purification (>99% purity, retention time 4.35 min., by analytical HPLC, 90%A-30 min.-100%B).

Peptide Hybrid 8: Isonip-Gly-Gly-Asp-Trp-OH



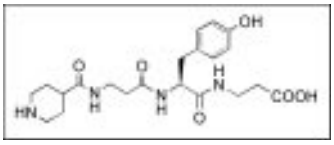
The Isonip-Gly-Gly-Asp-Trp-OH was synthesized using standard DuPont RamPS™ procedure starting from Fmoc-Wang resin. A white product (15.8 mg, 29% yield) was obtained after HPLC purification (95% purity, retention time 6.00 min., by analytical HPLC, 90%A-30 min.-100%B).

Peptide Hybrid 9: Isonip-Gly-Tyr-β-Ala-OH



The Isonip-Gly-Tyr-β-Ala-OH was synthesized using standard DuPont RamPS™ procedure starting from Fmoc-Wang resin. A white product (16.0 mg, 38% yield) was obtained after HPLC purification (98% purity, retention time 4.50 min., by analytical HPLC, 90%A-30 min.-100%B).

Peptide Hybrid 10: Isonip-β-Ala-Tyr-β-Ala-OH



The Isonip-β-Ala-Tyr-β-Ala-OH was synthesized using standard DuPont RamPS™ procedure starting from Fmoc-Wang resin. A white product (25.5 mg, 57% yield) was obtained after HPLC purification (97% purity, retention time 5.38 min., by analytical HPLC, 90%A-30 min.-100%B).

Results and Discussion

The designed antithrombotic agents were synthesized based on rational modifications of a lead peptide hybrid (Scheme 2). Arg-Gly-Asp-Phe-OH (RGDF-OH) was used as a standard and assumed to have a constant IC_{50} of 17 μM (ADP-activated platelet rich plasma or PRP/ADP).

The purity of the synthesized peptide hybrids was analyzed using HPLC. The molecular weight was calculated from the molecular formula and confirmed with FAB-MS. The inhibitory effects of the compound were determined by bioassays as IC_{50} values (concentration required for 50% inhibition of platelet aggregation). See Table 1.

Results. Compound **1** (IC_{50} = 0.050 μM, PRP/ADP) was the second most active analog among all the compounds tested. Replacing the C-terminal tyrosine-amide residue with tryptophan-amide led to further enhancement of activity in Compound **2** (IC_{50} = 0.031 μM, PRP/ADP). Compound **2** was approximately 550 times stronger than the standard peptide RGDF-OH. These compounds belong to the amidinobenzoyl series of compounds and are only marginally less active than the respective carboxyl terminal analogs.

Compounds **3**-**10** were synthesized containing the isonipecotyl group as a replacement of the amidinobenzoyl group. Several of these molecules exhibited good potency against platelet aggregation in the micromolar to nanomolar range of concentration and reflected well-defined and predictable structure-activity relationships. Compound **6** (IC_{50} = 0.254 μM, PRP/ADP) with C-terminal Trp residue demonstrated the highest inhibitory effect in this category. Compound **5** (IC_{50} = 2.10 μM, PRP/ADP) containing a C-terminal

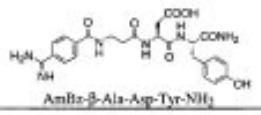
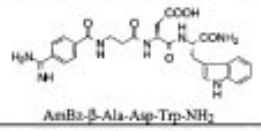
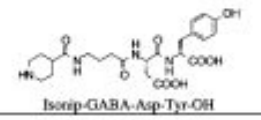
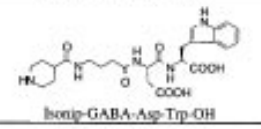
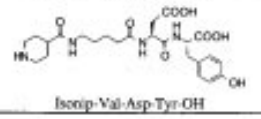
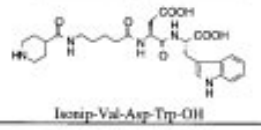
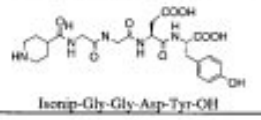
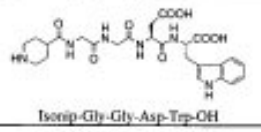
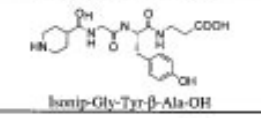
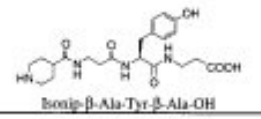
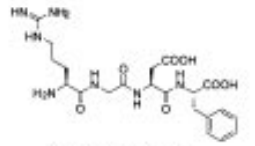
Compound Number	Structure	Molecular Weight	FAB-MS (M+1)	IC_{50} (μM, PRP/ADP)
1	 AmBz-β-Ala-Asp-Tyr-NH_2	512.202	513	0.050
2	 AmBz-β-Ala-Asp-Trp-NH_2	535.218	536	0.031
3	 Isonip-GABA-Asp-Tyr-OH	492.533	494	>50
4	 Isonip-GABA-Asp-Trp-OH	515.571	517	>50
5	 Isonip-Val-Asp-Tyr-OH	506.238	507	2.10
6	 Isonip-Val-Asp-Trp-OH	529.254	530	0.254
7	 Isonip-Gly-Gly-Asp-Tyr-OH	521.212	522	29.0
8	 Isonip-Gly-Gly-Asp-Trp-OH	544.569	546	4.05
9	 Isonip-Gly-Tyr-β-Ala-OH	420.469	421	>50
10	 Isonip-β-Ala-Tyr-β-Ala-OH	446.508	447	>50
Standard	 Arg-Gly-Asp-Phe-OH	493.217	494	17

Table 1. Peptide hybrid structure, molecular weight and inhibition of platelet aggregation *in vitro*.

Tyr residue was appreciably less active than its Trp analog **6**. Compound **8** (IC_{50} = 4.05 μM, PRP/ADP) was two times less active than **5**, while the Tyr analog of **8**, compound **7** (IC_{50} = 29.9 μM, PRP/ADP) was over 7 times less active than **8**. Compounds **3**, **4, 9** and **10** showed no activity within the measurable range.

Discussion. The use of peptide hybrids to mimic a known peptide sequence is well documented in scientific literature. Rational modifications of a lead structure can provide clues to the nature of interaction of a biomolecule with its receptor or enzyme, and may also lead to the development of biologically important drugs. This work describes a new series of potential antithrombotic peptide hybrids. The emphasis was on generating more varied structural information that may be useful for the design of an orally active platelet aggregation inhibitor. Besides providing interesting structure-activity correlations, this work has led to the synthesis of two extremely potent (low nanomolar concentrations) GPIIb/IIIa antagonists (**2**, IC_{50} = 0.031 μM, PRP/ADP; **1**, IC_{50} = 0.050 μM, PRP/ADP) in the amidinobenzoyl series. In the isonipecotyl series, a potent GPIIb/IIIa antagonist (**6**, IC_{50} = 0.254 μM, PRP/ADP) was discovered.

In general, the structure-activity relationships conform to the generalized three-point pharmacophoric hypothesis (Scheme 2) for GPIIb/IIIa antagonists. The results can be analyzed as follows.

1. Effect of C-terminal hydrophobic amino acids. Altering the C-terminal hydrophobic amino acid residue plays a major role in regulating activity as demonstrated in other systems.[4] Analogs containing a C-terminal tryptophan were, without exception, found to be more active than those containing tyrosine. Thus, **1** (IC_{50} = 0.050 μM, PRP/ADP) was 1.6 times less active than **2** (IC_{50} = 0.031 μM, PRP/ADP), **5** (IC_{50} = 2.10 μM, PRP/ADP) was about 8 times less active than **6** (IC_{50} = 0.254 μM, PRP/ADP) and **7** (IC_{50} = 29.0 μM, PRP/ADP) was about 7 times less active than **8** (IC_{50} = 4.05 μM, PRP/ADP). These results were consistent with results obtained in the N-terminal guanidinobenzoyl containing analogs.[7] The indole ring in tryptophan is larger than the 4-hydroxyphenyl ring in tyrosine. The larger volume of the indole ring perhaps increases the probability that it will adjust into the hydrophobic pocket of the receptor binding site by *induced fit*. The necessity of the bulkier hydrophobic group appears to decrease in the more active series as demonstrated by **1** which is only 1.6 times less active than **2.** This trend is actually reversed in the most active amidinobenzoyl series[7] in which the Tyr analog (USB-IPA-1305, IC_{50} = 0.020 μM, PRP/ADP, Scheme 2) is slightly more active than the corresponding Trp analog (USB-IPA-1302, IC_{50} = 0.026 μM, PRP/ADP).

2. Effect of varying linker size and nature. The linker length in the isonipecotyl series was adjusted to obtain maximum inhibition of platelet aggregation (Schemes 3 and 4). Having a six-atom linker between the isonipecotyl moiety and the aspartyl moiety was found to be optimal for activity. As a result, **5** (IC_{50} = 2.10 μM, PRP/ADP) was more potent than **3** (inactive), and **6** (IC_{50} = 254 μM, PRP/ADP) was more active than **4** (inactive). Compounds **5** and **6** contained the optimal six atom containing valeryl residue as a linker compared to **3** and **4**, which contained the five atom containing GABA residue (Scheme 4). In compounds **7** and **8** reasonable activity was observed which correlated with the presence of a similar six-atom tether represented by the Gly-Gly sequence.

There was an appreciably large difference in activities between compounds containing six-atom linkers of different kinds. The valeryl linker differs from the Gly-Gly linker in that the third and fourth atoms do not contain the conformationally constrained amide bond. The lower activity induced

by the Gly-Gly linker as opposed to the valeryl linker implied that the constraint of the amide bond is not well accepted by the inhibitor. Molecular modeling studies conducted on the amidinobenzoyl series[7] have pointed towards a preference for a curved backbone. The presence of the extra amide bond in the Gly-Gly linker should make the conformation more rigidly extended in nature, which may explain the loss in activity. Further studies may reveal interesting conformational preferences for the valeryl linker as opposed to the Gly-Gly linker and possibly to a more well-defined three-dimensional backbone structure in the overall pharmacophore concept.

3. Isonipecotyl group compared to the amidinobenzoyl as N-terminal residue. The substitution of the amidinobenzoyl group with the isonipecotyl group resulted in an overall loss in activity. The most active compound in the isonipecotyl series **6** (IC_{50} = 0.254 μM, PRP/ADP) was 10-fold less active than the corresponding analog in the amidinobenzoyl series. This result was not entirely surprising since the change from the amidinobenzoyl to the isonipecotyl is accompanied with rather drastic changes in the steric and electronic characteristics of the N-terminal group (Scheme 3). The isonipecotyl group contains the non-planar, non-aromatic six-membered piperidine ring compared to the planar, aromatic amidinobenzoyl group. The nitrogen in the isonipecotyl group is contained within the ring, while in the amidinobenzoyl group, the nitrogen exists outside the ring. Also, the isonipecotyl nitrogen should be distinctly more basic than the amidinobenzoyl nitrogen because of resonance effects. The electronic and conformational differences are bound to play an extremely important role in determining the activity of the inhibitor.

4. Replacement of the C-terminal carboxylic acid with an amide. The structural requirements at the C-terminus were investigated in the amidinobenzoyl series. Replacing the carboxylic acid terminus with an amide in the amidinobenzoyl series led to a slight decrease in activity as demonstrated in **1** (IC_{50} = 0.050 μM, PRP/ADP) as compared to USB-IPA-1305 (IC_{50} = 0.020 μM, PRP/ADP), and **2** (IC_{50} = 0.031 μM, PRP/ADP) as compared to USB-IPA-1302[7] (IC_{50} = 0.026 μM, PRP/ADP). The relatively small role played by the C-terminal carboxylic acid in regulating activity showed that it does not interact at any important secondary binding sites.

5. Testing the γ-turn hypothesis. Compounds **9** and **10** were designed as a preliminary experiment to test the γ-turn hypothesis (Scheme 6). These molecules were designed to have a tyrosyl group attached at a different point in the backbone, but in a way so that it would hypothetically occupy the same region in space as the hydrophobic group in the original pharmacophore (Scheme 6). Two different linker lengths were used in **9** and **10** to allow some variation for the proper fitting of the hydrophobic group. The aspartyl group side chain was mimicked by a β-alanine residue. However, both compounds were inactive. This may have been a result of large conformational changes induced by the replacement of the aspartyl residue and displacement of the tyrosyl residue so that the molecule no longer conformed to the three-dimensional conformation as represented by the three-point pharmacophore concept.

Conclusion. Through rational design of peptide hybrids, extremely potent inhibitors of platelet aggregation have been synthesized containing amidinobenzoyl and isonipecotyl groups as mimetics of the arginine side chain. These results provided some insight into the structural requirements for the binding of RGD mimetics to the platelet receptor GPIIb/IIIa.

In general, the activity of the reported peptide hybrids can be largely explained by the three-point pharmacophore model. With respect to the hydrophobic binding site, C-terminal tryptophan consistently gave better results than C-terminal tyrosine in the isonipecotyl series. The greater volume of the indole ring in Trp apparently allowed for a higher probability of interacting at the hydrophobic site possibly through an induced fit mechanism. A six-atom linker was confirmed to be optimum for activity (**5** and **6**). The negative test using GABA as a five-atom tether led to inactive compounds (**3** and **4**). Introducing conformational restriction in the six-atom tether in the form of an amide bond led to a great loss in activity. This serves as further evidence for a less extended and, possibly, curved backbone in the RGD pharmacophore model. It was revealed that the C-terminal carboxylic acid does not appear to play a significant role in regulating activity.

This research has revealed certain interesting facts about the nature of interaction at the ionic and hydrophobic sites as well as the nature of the backbone in the pharmacophore model. This structural information may be utilized in combination with molecular modeling to propose new designs for truly non-peptidic mimetics of the RGD sequence with potential oral activity.

Acknowledgments

I would first like to thank my parents and sister for their love and support. I would like to thank Professor Iwao Ojima for giving me the opportunity to work in his laboratory in the Department of Chemistry, SUNY at Stony Brook and for being supportive and kind. I would also like to thank Dr. Subrata Chakravarty for his help and advice by sharing his knowledge, generosity, and patience with me. Additionally, I would like to thank the rest of the research group for assisting me whenever the need arose. I also appreciate Dr. Peerschke and her group for providing the facilities for the bioassays.

I am grateful for everyone's help in making my research a truly great experience including my advisor, Mrs. Melanie Krieger, as well as my friends.

References

(1) Cook, N. S., G. Kottirsch, and H.-G. Zerwes. 1994. "Platelet glycoprotein IIb/IIIa antagonists." *Drugs of the Future*. 19: 135-59.

(2) Coller, B. S. 1991. "Platelets in cardiovascular thrombosis and thrombolysis." In: *The Heart and Cardiovascular System*, 2nd ed. (eds. Fozzard, H. A., E. Haber, R. B. Jennings, et al.) (New York: Raven Press): 219-73.

(3) Plow, E. F., S. E. D'Souza, and M. H. Ginsberg. 1992. "Ligand binding to GPIIb-IIIa: a status report." *Seminars in Thrombosis and Hemostasis*. 18: 324-32.

(4) Ojima, I., S. Chakravarty, and Q. Dong. 1995. "Antithrombotic agents: from RGD to peptide mimetics." *Bioorganic & Medicinal Chemistry.* 4: 337-60.

(5) Schiller, P. W. 1994. "Rational design of peptidomimetics: structural and pharmacological aspects." In: *Medicinal Chemistry for the 21st Century* (eds. Wermuth, C. G., N. Koga, H. Konig, et al.) (IUPAC/Blackwell Scientific Publications): 215-32.

(6) Eguchi, M., G. B. Crull, Q. Dong, et al. “Structure-activity relationship of small cyclic RGD peptides as inhibitors of platelet aggregation.” In: *The Peptides—Chemistry and Biology*, *Proceedings of the Thirteenth American Peptide Symposium* (eds. Hodges, R. S., and J. A. Smith) (Leiden: ESCOM): 780-1.

(7) Ojima, I., Q. Dong, E. Peerschke, et al. 1995. “Design, synthesis and SAR of RGD peptide hybrids as highly efficient inhibitors of platelet aggregation.” *Bioorganic and Medicinal Chemistry Letters.* 5: 1941-6.

(8) Chakravarty, S. 1995. *Progress Report 5.*