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DESIGN, SYNTHESIS AND SAR OF RGD PEPTIDE HYBRIDS AS HIGHLY EFFICIENT INHIBITORS OF PLATELET AGGREGATION

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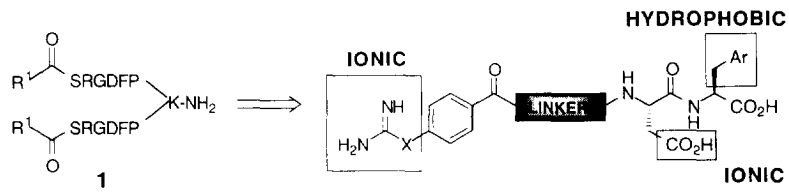
Abstract: A new series of peptide hybrids is developed as highly potent and selective antagonists of the GPIIb/IIIa receptor through rational modification of the RGDX sequence. Structure-activity relationships of these peptide hybrids have disclosed the important role of the C-terminal hydrophobic moiety and the N-terminal arginine side chain surrogates. Molecular modeling study strongly suggests the significance of a γ-turn conformation to achieve exceedingly high activity and receptor specificity.

Under pathophysiological conditions, the normal wound-healing process of platelet aggregation can become the cause of serious cardiovascular, cerebrovascular and peripheral vascular diseases.[1] Platelet aggregation is mediated by the platelet surface receptor, GPIIb/IIIa, that binds to the dimeric plasma glycoprotein, fibrinogen. The ligands of GPIIb/IIIa, including fibrinogen, are often characterized by the presence of a common RGD tripeptide recognition sequence.[2] The fibrinogen binding site(s) on GPIIb/IIIa can be competitively blocked by RGD peptides and their mimetics, making the RGD pharmacophore a suitable target for drug design and development.[3]

In the course of our study on rational modification of the RGD pharmacophore, we have reported double-strand RGD peptides (**1**) with enhanced antiplatelet activity compared to their single-strand counterparts.[4] We report here the simplification of these relatively large peptides into smaller peptide hybrids with very high affinity and selectivity for GPIIb/IIIa. The design, synthesis and SAR of these peptide hybrids are discussed on the basis of a three-point pharmacophoric binding model (Scheme 1).



Scheme 1. Three-point pharmacophoric model for RGD-based GPIIb/IIIa inhibitors.

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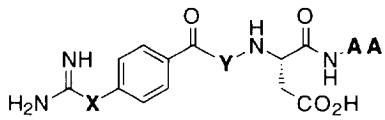
Synthesis of Hybrid Peptides. We have synthesized a new series of RGD peptide hybrids through solid-phase synthesis on Wang resin on the DuPont RamPS^{TM} system. Results are summarized in Table 1. (*S*)-Cyclohexylalanine was readily obtained through the hydrogenation of (*S*)-phenylalanine on 5% Rh-C in aqueous ethanol at 50 °C and 55 atm of hydrogen in an autoclave for 3 days. 4-Guanidinomethylbenzoic acid was prepared from 4-aminobenzoic acid by the literature method.[5] These amino acids were converted to their *N*-Fmoc derivatives by the standard method. The final step in the solid-phase synthesis involved the acylation of the N-termini with amidinobenzoic acid, guanidinobenzoic acid or guanidinomethylbenzoic acid in the presence of DIC and HOBt in DMF or DMF/DMSO. All hybrid peptides were obtained as trifluoroacetic acid (TFA) salt through cleavage from the resin using the standard deprotection procedure with TFA in the presence of thioanisole and ethanedithiol (EDT) or with bromotrimethylsilane (TMSBr).[6]

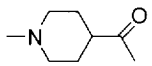
Bioassay *in vitro*. The hybrid peptides thus obtained were evaluated for their inhibitory activity against platelet aggregation *in vitro* by the standard assay method:[4] Fresh blood is taken from volunteers and placed in test tubes containing trisodium citrate. Platelet-rich plasma (PRP) is prepared by centrifugation and adjusted to 300,000/μL with platelet poor plasma (PPP). This PRP (450 μL) is placed in a cuvette equipped with a magnetic stirrer. Various concentrations of the peptide solution (36 μL) are added and incubated for 1 min, then platelet aggregation is induced by addition of ADP (54 μL, 100 μM). The extent of platelet aggregation is determined by a change in light transmission through the PRP on an aggregometer. The inhibitory ability is evaluated and the IC_{50} is determined as the concentration of a peptide hybrid required to produce 50% inhibition of the response to ADP in the presence of the TRIS buffer. In order to normalize the deviation in different sets of experiments, RGDF-NH_2 was employed as the standard (IC_{50} = 50 μM).

Structure-Activity Relationships. In the previous study,[4] we found that the activities of double-strand RGD peptides were significantly enhanced by N-terminal acylation, with the guanidinobenzoyl group being the most effective acyl moiety (**1**, USB-IPA-090, R^1 = guanidinobenzoyl, IC_{50} = 0.087 μM, PRP/ADP).[4] In an effort to reduce the molecular weight of the inhibitor and make the transition from peptides to peptide hybrids and eventually to non-peptide mimetics, we studied various fragments of **1** (USB-IPA-090) for activity. Our design strategy involved the following: (1) use of the guanidinobenzoyl moiety to entirely replace the arginine moiety in an RGDF-mimicking sequence; (2) adjustment of the distance between the guanidinobenzoyl moiety and the aspartyl side chain for optimum activity using adequate linkers; (3) use of different C-terminal aromatic groups in order to study a potential *localized* hydrophobic binding site, and (4) modification of the guanidinobenzoyl group.

The role of the linker was examined with analogs containing a guanidinobenzoyl group at the N-terminus and a Trp residue at the C-terminus. As Table 1 shows, β-Ala residue is superior to both Gly and γ-aminobutyric acid (GABA) residues as a linker (Compounds **2**-**4**). The marked difference between the β-Ala and GABA residues is also observed for analogs bearing an amidinobenzoyl group at the N-terminus (Compounds **16** and **21**). Accordingly, the β-Ala residue was chosen as the linker for further SAR study. It is of interest to note that **5** which contains a conformationally restricted GABA replacement is moderately active while the corresponding GABA analog **4** is virtually inactive. The flexibility of the linker in **4** may allow unacceptable backbone conformations to prevail over that required for binding to GPIIb/IIIa.

TABLE 1. Inhibition of Platelet Aggregation[a]: Effect of Linker Size, Arginine Side Chain Surrogates, and C-Terminal Amino Acid Side Chain



Compound	X	Y	AA	FAB-MS *m/z* $(M+1)^+$	IC_{50} (μM)[b]
2	NH	Gly	(*S*)-Trp	538	0.60
3 (USB-IPA-1102)	NH	β-Ala	(*S*)-Trp	552	0.070
4	NH	GABA[c]	(*S*)-Trp	566	> 50
5	NH		(*S*)-Trp	592	4.6
6	NH	Tyr	(*S*)-Trp	644	> 50
7	NH	β-Ala	(*S*)-Phe	513	0.390
8	NH	β-Ala	(*S*)-Tyr	529	0.190
9	NH	β-Ala	(*S*)-(4-F)Phe[c]	531	1.7
10 (USB-IPA-1106)	NH	β-Ala	(*S*)-(6H)Phe[c]	519	0.088
11	NH	β-Ala	(*S*)-1-Nal[c]	563	0.77
12	NH	β-Ala	(*S*)-2-Nal[c]	563	0.32
13	NH	β-Ala	(*RS*)-β-Phe[c]	513	6.1
14	NH	β-Ala	(*S*)-Phg[c]	499	2.9
15	$NHCH_2$	β-Ala	(*S*)-Trp	566	0.71
16 (USB-IPA-1302)	-	β-Ala	(*S*)-Trp	537	0.026
17 (USB-IPA-1305)	-	β-Ala	(*S*)-Tyr	514	0.020
18	-	β-Ala	(*R*)-Tyr	514	1.2
19 (USB-IPA-1303)	-	β-Ala	(*S*)-2-Nal[c]	548	0.044
20 (USB-IPA-1304)	-	β-Ala	(*S*)-(4-F)-Phe[c]	516	0.047
21	-	GABA[c]	(*S*)-Trp	551	> 50

[a]Human platelet aggregation was induced by ADP. [b]Normalized IC_{50} value based on the IC_{50} value of RGDF-NH_2 equal to 50 μM. Each value is the average of three runs and experimental error is within 15%. [c]GABA = γ-amino butanoic acid; (4-F)-Phe = 4-fluorophenylalanine; (6H)Phe = cyclohexylalanine; Nal = naphthylalanine; β-Phe = 3-phenyl-β-alanine; Phg = phenylglycine.

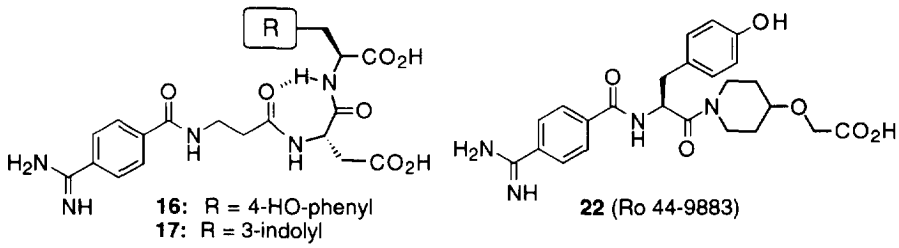
The amidinobenzoyl moiety was found to be the most effective N-terminal acyl group when compared to the guanidinobenzoyl and the guanidinomethylbenzoyl moieties (Compounds **3**, **15**, and **17**). Thus, **16** (USB-IPA-1302) is almost 3 times as active as **3** and about 30 times more active than **15**. As Table 1 shows, the *N*-amidinobenzoyl analogs with the β-Ala linker are uniformly more active than the corresponding *N*-guanidinobenzoyl analogs, although the gradation in activity between analogs is not necessarily parallel (*vide infra*).

Next, the effects of different C-terminal hydrophobic amino acid residues on the potentiation of activity were investigated. In both *N*-guanidinobenzoyl and *N*-amidinobenzoyl series, the (*S*)-Trp analogs, **3** and **16**, are extremely active. The (*S*)-Tyr analog **17** (USB-IPA-1305)[7] in the *N*-amidinobenzoyl series is also exceedingly active. It should be noted that **16** and **17** are among of the most potent GPIIb/IIIa antagonists ever reported.[7] While **17** is slightly more potent than the (*S*)-Trp analog **16** in the *N*-amidinobenzoyl series, the (*S*)-Tyr analog **8** in the *N*-guanidinobenzoyl series is 3 times *less* active than the corresponding (*S*)-Trp analog **3**. Also, it is noteworthy that the (*R*)-Tyr analog **18** is 60 times less active than the (*S*)-Tyr counterpart **17**, which indicates rather strict requirement for the proper spatial arrangement of the hydrophobic moiety to achieve excellent activity(*vide infra*). Aromaticity does not appear to be essential for this hydrophobic interaction since the cyclohexylalanine analog **10** is 4.5 times more active than the corresponding aromatic analog **7** in *N*-guanidinobenzoyl series. The location of the hydrophobic substituent at the C-terminus is important, thus the introduction of β-Phe and Phg residues to the C-terminus leads to a substantial loss in activity (Compounds **13** and **14**).

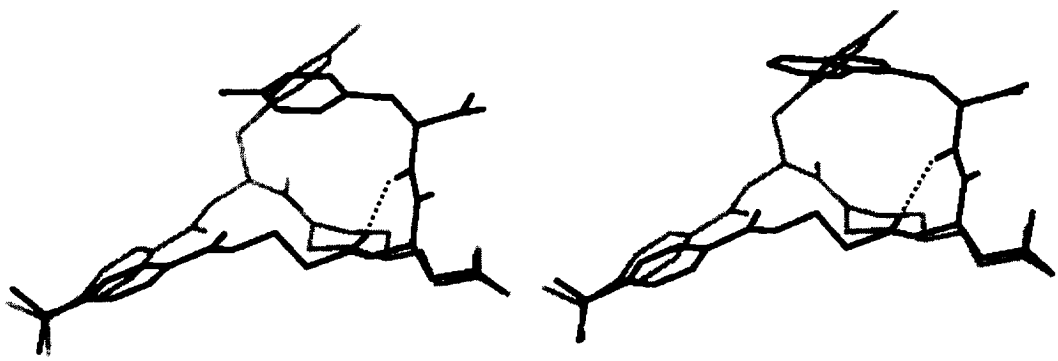
Receptor Specificity. Peptide hybrids **16** and **17** are found to be not only exceedingly active antagonists of GPIIb/IIIa, but also extremely selective agents to GPIIb/IIIa in comparison with the closely related integrins, vitronectin receptor ($\alpha_v\beta_3$) and fibronectin receptor ($\alpha_5\beta_1$). In a solid-phase competitive [^{3}H]-SK&F-107260 (***SK***)[8a] – receptor binding assay to the purified immobilized GPIIb/IIIa ($\alpha_{IIb}\beta_3$) and $\alpha_v\beta_3$ receptors,[9] **17** showed extremely high affinity to GPIIb/IIIa and a 35,700-fold lowered affinity to $\alpha_v\beta_3$ (K_i = 2.1 ± 0.4 nM, ****SK***-GPIIb/IIIa binding assay; K_i = 75 ± 2.5 μM, ****SK***-$\alpha_v\beta_3$ binding assay). Peptide hybrid **16** showed a 15,200-fold selectivity to GPIIb/IIIa over $\alpha_v\beta_3$ in the same assay (K_i = 2.3 ± 0.1 nM, ****SK***-GPIIb/IIIa binding assay; K_i = 35 ± 1.5 μM, ****SK***-$\alpha_v\beta_3$ binding assay). These selectivity values are comparable to those for **22** (Ro 44-9883), a potent GPIIb/IIIa inhibitor in phase II clinical trials, and its analogs as reported by Alig *et al.*[10] Both pepide hydrids **16** and **17** also failed to interact with the purified, immobilized fibronectin receptor ($\alpha_5\beta_1$) at concentrations up to 100 μM, i.e., **16** and **17** are *at least* 43,500 and 47,600 times, respectively, more selective to GPIIb/IIIa than $\alpha_5\beta_1$.[11]

A Common Hydrophobic Binding Site: Molecular Modeling Studies. Conformational studies were performed using Biosym 2.35 and Sybyl 6.04 software. Although the existence of a possible hydrophobic binding site has been alluded in different systems,[8] no particular investigation has been carried out, to the best of our knowledge, to establish its precise position in the pharmacophoric model. We believe that the correct positioning of the hydrophobic moiety is very important for the design of extremely active non-peptide GPIIb/IIIa antagonists. Our molecular modeling and the SAR studies (*vide supra*) point toward a *localized* hydrophobic binding site. Low-energy conformations of **16** and **17** obtained from comparative molecular dynamics simulations in consensus with those of other known GPIIb/IIIa antagonists have indicated that the hydrophobic moiety tends to orients itself on the convex side of the slightly curved backbone. In particular, comparison with **22** shows reasonable overlap of the hydrophobic moieties together with two ionic moieties at the N- and C-termini (Figure 1). It is strongly suggested that the binding conformations of **16** or **17** are stabilized by a γ-turn[12] caused by a hydrogen bonding between the amide hydrogen of the C-terminal residue with the carbonyl oxygen of the β-Ala residue that appears to position the hydrophobic moiety precisely into the binding pocket (Scheme 2).

As mentioned above, the (*S*)-Tyr analog **17** is 60 times more potent than the (*R*)-Tyr analog **18**. The γ-turn stabilization should make the spatial orientation of the hydroxyphenyl group in **17** and **18** quite different, which can account for the observed marked loss in activity. It is a noteworthy fact that **6** that contains a Tyr linker and a (*S*)-Trp C-terminal is completely inactive. Molecular dynamics studies on this molecule suggest that neither aromatic ring is able to occupy the region in space which corresponds to the postulated hydrophobic site.



Scheme 2. The proposed γ-turn in **16** and **17** and the orientation of the hydrophobic group (R) in comparison with that of **22**.



(a) (b)

Figure 1. (a) Overlay of **16** and **22**; (b) Overlay of **17** and **22**.

Further studies on the design and synthesis of non-peptide mimetics based on **16** and **17** are actively underway.

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References:

1. Coller, B. S. In *The Heart and Cardiovascular System, Second Ed.;* Fozzard, H. A.; Haber, E.; Jennings, R. B.; Katz, A. M.; Morgan, H. E., Eds.; Raven: New York, 1992; pp. 219-273.
2. (a) Ruoslahti, E.; Pierschbacher, M. D. *Science* **1987**, *238*, 491. (b) Ruoslahti, E.; Pierschbacher, M. D. *Cell* **1986**, *44*, 517. (c) Pierschbacher, M. D.; Ruoslahti, E. *Nature* **1984**, *309*, 30.
3. (a) Ojima, I.; Chakravarty, S.; Dong, Q. *Bioorg. Med. Chem.* **1995**, *3*, 337 and references cited therein. (b) Ojima, I.; Dong, Q.; Chakravarty, S. *J. Synth. Org. Chem. (Jpn)* **1994**, *52*, 413. (c) Cook, N. S.; Kottirsch, G.; Zerwes, H. -G. *Drugs Future* **1994**, *19*, 135. (d) Weller, T.; Alig, L.; Muller, M. H.; Kouns, W. C.; Steiner, B. *Drugs Future* **1994**, *19*, 461. (e) Blackburn, B. K.; Gadek, T. R. *Ann. Rep. Med. Chem.* **1993**, *28*, 79.
4. Ojima, I.; Dong, Q.; Eguchi, M.; Oh, Y. -I.; Amann, C. M.; Coller, B. S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1749.
5. Miller, A. E.; Bischoff, J. J. *Synthesis*, **1986**, 777.
6. Yajima, H.; Fujii, N.; Funakoshi, S.; Watanabe, T.; Murayama, E.; Otaka, A. *Tetrahedron*, **1988**, *44*, 805.
7. After this work had been completed, a patent including the same structure as **17** (USB-IPA-1305) in its claims was disclosed. See Alig, L.; Edenhofer, A.; Müller, M.; Trzeciak, A.; Weller, T. *U. S. Patent*, 5,273,982, 1993.
8. (a) Ali, F. E.; Bennett, D. B.; Calvo, R. R.; Elliott, J. D.; Hwang, S. -M.; Ku, T. W.; Lago, M. A.; Nichols, A. J.; Romoff, T. T.; Shah, D. H.; Vasko, J. A.; Wong, A. S.; Yellin, T. O.; Yuan, C. -K.; Samanen, J. M. *J. Med. Chem.* **1994**, *37*, 769. (b) Charon, M. H.; Poggi, A.; Donati, M. B.; Marguerie, G. *Peptides: Chemistry, Structure and Biology. Proceedings of the Eleventh American Peptide Symposium*, Rivier, J. E.; Marshall, G. R., Eds.; ESCOM: Leiden, 1990; pp 82-83. (c) Dong, Q.; Chakravarty, S.; Ojima, I. *208th American Chemical Society National Meeting*, 21-25 August 1994; *Abstracts* **1994**, MEDI 60. (d) Ku, T. W.; Ali, F. E.; Barton, L. S.; Bean, J. W.; Bondinell, W. E.; Burgess, J. L.; Callahan, J. F.; Calvo, R. R.; Chen, L.; Eggleston, D. S.; Gleason, J. G.; Huffman, W. F.; Hwang, S. M.; Jakas, D. R.; Karash, C. B.; Keenan, R. M.; Kopple, K. D.; Miller, W. H.; Newlander, K. A.; Nichols, A.; Parker, M. F.; Peishoff, C. E.; Samanen, J. M.; Uzinskas, I.; Venslavsky, J. W. *J. Am. Chem. Soc.* **1993**, *115*, 8861. (e) Scarborough, R. M.; Naughton, M. A.; Teng, W.; Rose, J. W.; Phillips, D. R.; Nannizzi, L.; Arfsten, A.; Campbell, A. M.; Charo, I. F. *J. Biol. Chem.* **1993**, *268*, 1058. (f) Scarborough, R. M.; Rose, J. W.; Naughton, M. A.; Phillips, D. R.; Nannizzi, L.; Arfsten, A.; Campbell, A. M.; Charo, I. F. *J. Biol. Chem.* **1993**, *268*, 1066. (g) Tranqui, L.; Andrieux, A.; Hurdy-Clergeon, G.; Ryckewaert, J.-J.; Soyez, S.; Chapel, A.; Ginsberg, M. H.; Plow, E. F.; Marguerie, G. *J. Cell Biol.* **1989**, *108*, 2519.
9. Test compounds were assayed for their abilities to compete for the binding to GPIIb/IIIa ($\alpha_{IIb}\beta_3$) or to $\alpha_v\beta_3$ with [^{3}H]-SK&F-107260, a cyclic Arg-Gly-Asp-containing pentapeptide. The specific activity of [^{3}H]-SK&F-107260 was 65-80 Ci/mmol. The receptors $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ were purified from human platelets and were immobilized on the 96-well microtiter plate. Test compounds of various concentrations (0.0001-100 μM) were added to the wells precoated with the integrin receptors, followed by the addition of 4.5 nM of [^{3}H]-SK&F-107260. The samples were incubated for 1 hour at room temperature. Receptor bound [^{3}H]-SK&F-107260 was determined by liquid scintillation counting. The IC_{50} (concentration of the test compound to inhibit 50% binding of [^{3}H]-SK&F-107260) was determined by a non-linear, least squares curve-fitting routine, which was modified from the LUNDON-2 program. The K_i (dissociation constant of the test compound) wsa calculated according to the following equation: $K_i = IC_{50}/(1 + L/K_d)$, where L and K_d were the concentration and the dissociation constant of [^{3}H]-SK&F-107260, respectively. In the screening assay, K_d was determined as 2.25 nM and L was 4.5 nM. The K_i values were the mean ± S.D. from three experiments, each with quadruplicate determinations.
10. Alig, L.; Edenhofer, A.; Hadvary, P.; Hurzeler, M.; Knopp, D.; Muller, M.; Steiner, B.; Trzeciak, A.; Weller, T. *J. Med. Chem.* **1992**, *35*, 4393.
11. The fibronectin receptor ($\alpha_5\beta_1$) was purified from human placenta and was immobilized on the 96 well microtiter plates. Test compounds were assayed for their abilities to compete with the binding to $\alpha_5\beta_1$ with 1 nM of biotinylated-fibronectin antibody conjugated to alkaline phosphatase, followed by the addition of *p*-nitrophenylphosphate. Absorbance at 405 nm was determined using a microtiter plate reader.
12. Analogous turns have been observed in RGD-containing cyclic peptides and the information has been used for the design of backbone mimetics,[8d] see Peishoff, C. E.; Ali, F. E.; Bean, J. W.; Calvo, R. R.; D'Ambrosio, C. A.; Eggleston, D. S.; Hwang, S. M.; Kline, T. P.; Koster, P. F.; Nichols, A.; Powers, D.; Romoff, T.; Samanen, J. M.; Stadel, J.; Vasko, J. A.; Kopple, K. D. *J. Med. Chem.* **1992**, *35*, 3962.

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