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# DESIGN AND SYNTHESIS OF NEW ANTAGONIST PEPTIDES FOR PLATELET GPIIb/IIIa RECEPTOR AS ANTI-THROMBOTIC AGENTS

Yoshio Hayashi*, Yoshimi Sato, Jun Katada, Yoshimi Takiguchi, Iwao Ojima[¶] and Isao Uno

*Life Science Research Center, Advanced Technology Research Laboratories, Nippon Steel Corporation, 1618 Ida, Nakahara-ku, Kawasaki 211, Japan*

[¶]*Department of Chemistry, State University of New York at Stony Brook, NY 11794-3400*

**Abstract:** The structure-activity relationships of N-terminal modified RGD peptides against platelet aggregation inhibitory activity were studied, and Orotyl-Ser-Arg-Gly-Asp-Trp (NSL-9403) was found to be a new and effective anti-thrombotic agent during extracorporeal circulation. Copyright © 1996 Elsevier Science Ltd

Contact between blood and an artificial surface during extracorporeal circulation causes undesired platelet aggregation to induce various haemostatic defects. Especially profound platelet loss and platelet dysfunction are suspected to be the major causes of the bleeding.[1] Also, the formation of micro thrombi observed during extracorporeal circulation is responsible for emboli in lung, brain, and other organs.[2] Such undesired platelet activation and aggregation are induced by the interaction between the platelet fibrinogen receptor and fibrinogen-coated surface of the membrane oxygenator.[3]

The binding of adhesive proteins such as fibrinogen or von Willebrand factor to the platelet fibrinogen receptor, glycoprotein (gp) IIb/IIIa, on activated platelets is the critical event leading to platelet aggregation.[4] GpIIb/IIIa recognizes the arginine-glycine-asparatic acid (RGD) sequence of fibrinogen, which is the common recognition sequence in the integrin family. These small RGD peptide derivatives possess the ability to inhibit fibrinogen binding to activated platelets and then platelet aggregation.[5] Thereafter, many compounds including the RGD sequence and non-peptide RGD mimetics, have been synthesized as anti-platelet aggregatory agents.[6] One of the problems with such compounds is to prolong the bleeding time *in vivo* at higher doses,[7] whereas the fibrinogen antagonists efficiently prevent the platelet loss in an extracorporeal circulation model.[8]

Thus the ideal fibrinogen antagonist should fulfill such conditions as (1) the agent must be a potent gpIIb/IIIa antagonist, (2) the agent is stable in extracorporeal circuits, and (3) the agent easily decomposes *in vivo* with no prolonged postoperative bleeding.

Peptides are possible candidates for fulfilling such conditions since peptides are easily digested into non-toxic inactive forms *in vivo*. From the preliminary study of RGD peptides, SRGDW($IC_{50}$ = 40μM) was chosen as our lead compound. In this paper we describe the design and SAR study of novel RGD peptides for their platelet aggregation inhibitory activity in extracorporeal circulation.

## Chemistry

All the peptides were synthesized by solid-phase methods using a 9-fluorenylmethoxycarbonyl amino acid

on an Applied Biosystems, Inc. 431A peptide synthesizer. The final step in the solid phase synthesis involved the acylation of N-termini with different types of carboxylic acids in the presence of DCI and HOBt in DMF. A trimethylsilylbromide - thioanisole system was used for the final deprotection.[9] The crude peptides were purified by preparative HPLC. All the peptides were characterized by FAB-MASS, amino acid analysis, and analytical HPLC. The purified peptides were at least 98% pure.

**Structure-Activity Relationships**

The SAR study was carried out for a series of N-terminal modified SRGDW peptides, **1-24**, to elucidate the factors that effect the platelet aggregation inhibitory activity.[10] It has been shown[11] that N-terminal amino group modification stabilizes RGD analogs in plasma without further modification of the C-terminal carboxyl group. Thus, we decided to leave the C-terminal of peptides **4-24** unprotected. In the N-terminal modification, we focused on two factors, i) hydrophobicity and ii) hydrogen bonding, since hydrophobic interaction and hydrogen bonding should be the major factors in peptide-protein interaction.

First, the effect of hydrophobicity was studied. In peptides **2-5**, highly hydrophobic groups, phenyl and phthalimide groups, were introduced to the N-terminal of SRGDW sequence. As Table 1 shows, these peptides possess no significant difference in inhibitory activity compared to SRGDW.

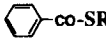
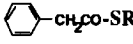
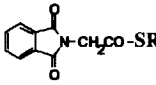
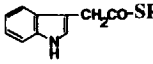
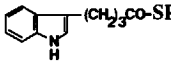
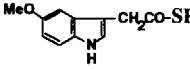
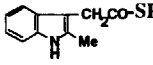
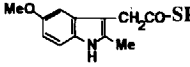
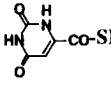
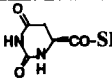
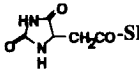
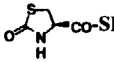
Next, in peptides **6-11**, the effect of hydrogen bonding was studied by introducing an indole unit which tends to form a strong hydrogen bond. Peptides **6** and **9** showed 6 and 4 times higher activity than that of SRGDW, respectively. This indicates that the hydrogen bonding ability is a more important factor than hydrophobicity for improving the activity of these peptides.

To enhance hydrogen bonding with the receptor, an orotyl group, an aromatic group with high hydrophilicity, was introduced. This peptide **12** (NSL-9403) turned out to be 10 times as active as SRGDW. It should be noted that peptide **13** with the dihydroorotyl group was also 5 times as active as SRGDW, indicating the insignificance of the aromaticity factor.

Encouraged by this result, we synthesized a series of the peptides containing different types of N-terminal hydrophilic cyclo-acyl groups, i.e., L-pyroglutamyl (peptide **14**), 5-hydantoinacetyl (peptide **15**), (-)-2-oxo-4-thiazolidinecarbonyl (peptide **16**), L-thioprolyl (peptide **17**), *trans*-4-hydroxy-L-prolyl (peptide **18**), L-prolyl (peptide **19**). Thiazolidine containing peptides, **16** and **17**, were 3 and 7 times less active than peptide **12**, respectively. Peptide **18** bearing the *trans*-4-hydroxy-L-prolyl group was also 7 times less active than peptide **12**. However, L-pyroglutamyl and hydantoinacetyl containing peptides, **14** and **15**, retain substantially the same inhibitory activity as peptide **12**. Moreover, peptide **19** bearing N-L-prolyl group possesses even better inhibitory activity ($IC_{50}$ = 0.87μM) that is 46-fold more potent than SRGDW and the best in this series.

Peptide **20** bearing N-D-prolyl group was 38 times less active than the corresponding L-isomer, peptide **19**. This indicates the importance of the L-configuration of the proline residue. The free secondary amino moiety is also significant for high inhibitory activity: When the imino group was modified (1) by the introduction of the acetyl or methyl group (peptides **21** and **22**) or (2) by the replacement of a nitrogen atom with an oxygen atom (peptide **25**), the inhibitory activity decreased 20 to 60 times compared to that of peptide **19**. Moreover, the change of ring size (the nitrogen-heterocycle) from 5 to 6 in peptide **23** decreased its activity, though peptide **24** with a 4-membered ring kept half of its activity. These results suggest the ionic interaction with the receptor at the N-terminal of the peptide besides hydrogen bonding and the strict spatial arrangement of the amino group are

**TABLE 1. Inhibition of human platelet aggregation[a]**

| Compound | Structure | FAB-MS m/z(M+1)+ | $IC_{50}$ (μM) [b] |
|---|---|---|---|
| 1 | N-Ac-SRGDW-$NH_2$ | 662 | 74 |
| 2 |  | 723 | 34 |
| 3 |  | 737 | 59 |
| 4 |  | 806 | 21 |
| 5 | Phe-SRGDW-OH | 767 | 23 |
| 6 | Trp-SRGDW-OH | 806 | 6.8 |
| 7 |  | 777 | 21 |
| 8 |  | 805 | 21 |
| 9 |  | 807 | 11 |
| 10 |  | 791 | 22 |
| 11 |  | 821 | 25 |
| 12 |  (NSL-9403) | 758 | 4 |
| 13 |  | 760 | 8.7 |
| 14 | Pyr-SRGDW-OH | 731 | 5.6 |
| 15 |  | 760 | 6.9 |
| 16 |  | 749 | 12 |
| 17 |  | 735 | 27 |
| 18 | Hyp-SRGDW-OH | 733 | 28 |
| 19 | Pro-SRGDW-OH | 717 | 0.87 |
| 20 | D-Pro -SRGDW-OH | 717 | 33 |
| 21 | N-Ac-Pro-SRGDW-OH | 760 | 50 |
| 22 | N-Me-Pro-SRGDW-OH | 731 | 20 |
| 23 |  | 731 | 11 |
| 24 |  | 703 | 1.3 |
| 25 |  | 718 | 21 |

[a]Human platelet aggregation was induced by collagen. [b]Normalized $IC_{50}$ value of SRGDW equal to 40μM. Each value is the average of three runs and experimental error is within 20 %. The $IC_{50}$ value of RGDF is 50μM.

required to achieve excellent activity.

### Stability of the synthetic peptides in human plasma

To select the compounds which are stable in extracorporeal circuits, but can easily be decomposed *in vivo* without prolonged postoperative bleeding, the stability of five selected peptides was examined in human plasma (*in vitro*) and mice (*in vivo*).[12] As Fig. 1 shows, the peptides whose N-terminal units have free amino groups are degradable in plasma. The $T_{1/2}$ of peptide **6** was 11 min, while the most potent peptide **19** ($T_{1/2}$ = 74 min) lasted somewhat longer than peptide **6**. On the other hand, peptides **12** and **14** that have masked N-terminal units, i.e., orotyl and pyroglutamyl groups, respectively, are extremely stable in plasma and their $T_{1/2}$ values are longer than 240 min. However, these peptides were degraded within 10 min in mice when they were intravenously administered. Hence we selected peptide **12**, **NSL-9403**, as the candidate for the new anti-thrombotic agent.

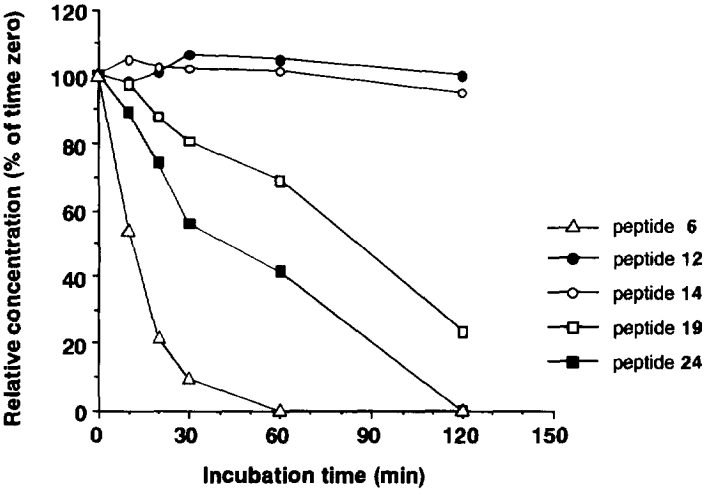



**Fig. 1 Stability of the peptides in human plasma during an incubation at 37 °C.**
The concentration of each peptide without incubation at 37 °C was 100% and the peptide concentration at each time point was represented by relative value.

### Guinea pig arterio-venous shunt model

To examine an anti-thrombotic activity of peptide **12**, **NSL-9403**, *in vivo*, a guinea pig arterio-venous shunt model, in which platelet-rich thrombus is formed around a silk thread in an extracorporeal shunt circuit, was used.[13] As Fig. 2 shows, the total thrombus weight over 60 min was 33.9 ± 4.7 mg in the control group where saline was infused. Infusion of heparin (100 units/kg/hr), which is an inhibitor of blood coagulation but does not inhibit platelet aggregation at this dose, inhibited the thrombus formation around the thread, but not completely (total wet weight during the 60 min infusion period was 13.9 ± 2.2 mg). Intravenous infusion of peptide **12** inhibited the thrombus formation in a dose-dependent manner above 10 mg/kg/hr. After the

termination of the infusion, the thrombus formation was examined for another 60 min. Though the inhibition of thrombus formation by heparin continued for at least 60 min after the termination of its infusion, the anti-thrombotic activity of **NSL-9403** disappeared quickly and the thrombus weight formed during this 60 min post-infusion period did not show a significant difference from that of the control group even after the administration of 100 mg/kg/hr (35.2 ± 3.3 mg in the control group versus 29.6 ± 5.3 mg in the 100 mg/kg/hr group). These results suggest that **NSL-9403**, when it is intravenously infused, exerts an anti-thrombotic activity in an extracorporeal circuit, without unfavorable postoperative bleeding.

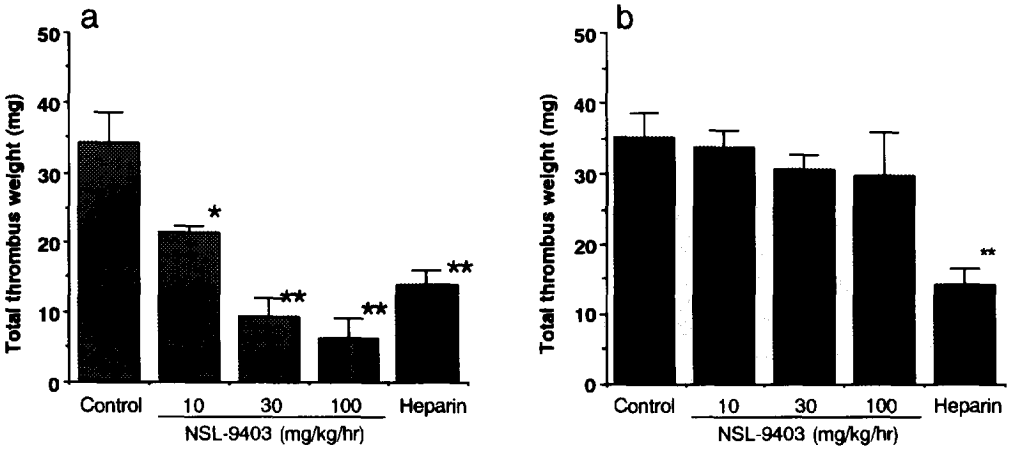



**Fig. 2 An anti-thrombotic activity of NSL-9403 in a guinea pig arterio-venous shunt model.**

NSL-9403 (10, 30 and 100 mg/kg/hr) or heparin (100 units/kg/ml) was infused via a cannula at a right jugular vein and the weight of the thrombi formed during the infusion for 60 min (a) and during a post-infusion period for 60 min (b) were measured. Mean ± SEM, n=5; * $P < 0.05$, ** $P < 0.01$: significantly different from the control.

## Conclusion

We found potent RGD peptide derivatives as new anti-thrombotic agents for extracorporeal circulation based on the SAR study of the N-terminal modified analogs of SRGDW by considering three factors in ligand-receptor interaction, 1) hydrophobic interaction, 2) hydrogen bonding, and 3) ionic interaction. The addition of an ionic dipolar interaction site derived from the amino group of PSRGDW was the most effective for achieving high inhibitory activity ($IC_{50}$ = 0.87μM). However, this peptide was unstable under the condition of extracorporeal circulation. The addition of the N-Orotyl group to SRGDW resulted in a potent ($IC_{50}$ = 4.0μM) derivative, **NSL-9403**, which is stable ($T_{1/2}$ > 4 h) in human plasma, but is short acting in the body (mice). These biological characteristics of **NSL-9403** satisfy our requirements for the new anti-thrombotic agent in extracorporeal circulation. The pharmacological details of **NSL-9403** will be published elsewhere.[14]

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12. Stability of the synthetic peptides in human plasma, anti-coagulated with 0.38% trisodium citrate, was measured. Each peptide was dissolved in phosphate-buffered saline (pH 7.4), and 100 μl of these peptide solutions were then mixed with 900 μl of the human plasma and incubated at 37 °C. A part of the plasma was sampled periodically and the concentration of each peptide was measured by reverse-phase HPLC ($C_{18}$ analytical column; 4.6 mm x 150 mm). To examine the stability of the peptides *in vivo*, each peptide was administered intravenously (1mg/kg) via a tail vein of ICR mice. Plasma concentrations of the peptides at various time points were determined by HPLC analysis as mentioned above.
13. Male hartley guinea pigs weighing 230 - 330 g were anesthetized with pentobarbital (35 mg/kg, i.p.). Polyethylene tubes filled with saline containing 100 units/ml heparin were then inserted into the left jugular vein and the right carotid artery and these two tubes were connected with a silicon tube filled with the same solution. The whole length of this shunt was 6 cm. Anti-thrombotic agents were infused via a cannula at the right jugular vein at the rate of 2 ml/hr for 70 min. Ten min after the onset of the infusion, a silk thread was inserted into the shunt. Every 20 min a silk thread was replaced and its wet weight was measured. After the termination of the infusion, the measurement of the thread weight every 20 min was continued for another 60 min. The thrombus weight was calculated from the wet weight of the thread before and after the experiment, and the total thrombus weight was the sum of three thrombus weights obtained during the infusion period or post-infusion period.
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