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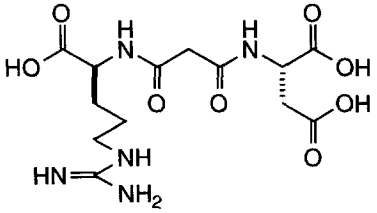
Synthesis and Biological Properties of Partially Modified Retro and Retro-inverso Pseudo Peptides of Arg-Gly-Asp (RGD)

Naoyuki Nishikawa,*† Hiroyuki Komazawa,† Atsushi Orikasa,† Mitsuo Yoshikane,† Jiro Yamaguchi,† Masayoshi Kojima,† Mitsunori Ono,† Isamu Itoh,† Ichiro Azuma,†† Hideki Fujii,††† Jun Murata††† Ikuo Saiki,†††

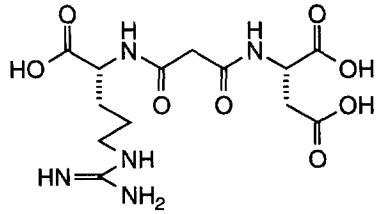
† *Ashigara Research Laboratories, Fuji Photo Film Co., Ltd., Minamiashigara, Kanagawa 250-01, Japan*
†† *Institute of Immunological Science, Hokkaido University, Sapporo, Hokkaido 060, Japan*
††† *Department of Pathogenic Biochemistry, Research Institute for WAKAN-YAKU, Toyama Medical Pharmaceutical University, Sugitani, Toyama 930-01, Japan*

Abstract: Partially modified retro and retro-inverso peptide analogs of Arg-Gly-Asp (RGD) were synthesized and examined their inhibitory effects on experimental lung metastasis of murine melanoma and adenosine 5'-diphosphate (ADP) induced platelet aggregation. The analogs showed efficient therapeutic potency for the tumor metastasis but low inhibitory effect on ADP induced platelet aggregation.

Interaction of tumor cells with extracellar matrix plays an important role in the complex process of cancer metastasis. The Arg-Gly-Asp (RGD) sequence is a cell binding unit of adhesion molecules and extracellar matrix components such as fibronectin, vitronectin and fibrinogen, and recognized by integrin receptors on the cell surface. Humphries et al. have reported that synthetic oligopeptide containing the RGD sequence inhibited metastasis of murine melanoma.[1] However, high dose of the peptide was required for effective inhibition of the metastasis because of the rapid degradation and clearance *in vivo*. To overcome this problem, we developed conjugations of cell-adhesive peptides with various drug carriers such as poly (ethylene glycol)[2] or chitin derivatives.[3] We also reported that liposomes having RGD related peptides on the surface showed high anti-metastatic efficacy.[4] Recently, syntheses of the pseudo peptides have been studied extensively to avoid enzymatic degradation *in vivo*. Since the Arg-Gly linkage of the sequence has been reported as a easy degradable linkage,[5] we designed the partially modified retro and retro-inverso pseudo peptides[6] of the RGD peptide (**1a** and **1b**) reversing amide bond between arginyl and glycyl residue. The RGD related peptides are also concerned with platelet aggregation. In this paper, we wish to describe the synthesis, the anti-metastatic effects and the anti-platelet aggregation activities of the pseudo peptides.



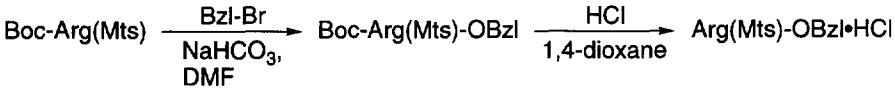
1a: Partially modified retro peptide



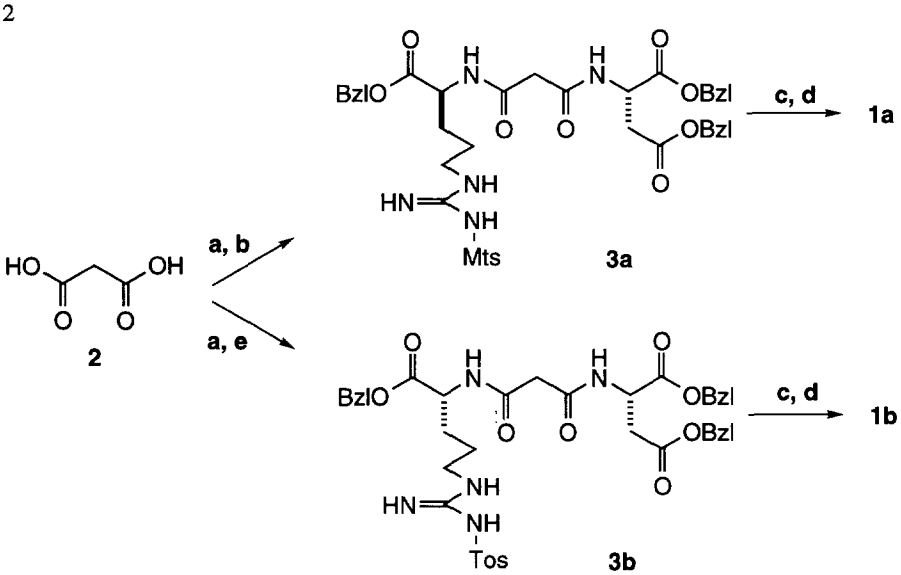
1b: Partially modified retro-inverso peptide

N^{ω}-2,4,6-trimethylbenzenesulfonyl-L-arginine benzyl ester hydrochloride (Arg(Mts)-OBzl•HCl) was prepared from N^{α}-Boc-N^{ω}-2,4,6-trimethylbenzenesulfonyl-L-arginine (Boc-Arg(Mts)) by using general method (Scheme 1). N^{ω}-toluensulfonyl-D-arginine benzyl ester hydrochloride (D-Arg(Tos)-OBzl·HCl) was prepared by similar way using N^{α}-Boc-N^{ω}-toluensulfonyl-D-arginine (Boc-D-Arg(Tos)). Scheme 2 shows synthetic routes of the pseudo peptides. Malonic acid (**2**) was converted to protected pseudo peptides (**3a** and **3b**) by sequential peptide couplings using diphenylphosphorylazide (DPPA)[7] in DMF at room temperature. After purification using silica gel chromatography (eluent: AcOEt), the protected pseudo peptides were treated with 1M trifluoromethanesulfonic acid (TFMSA)-thioanisole-*m*-cresol / TFA solution[8] to remove all protecting groups. The products were passed over an ion exchange column (Amberlite® IRA-400 (Cl^-)), then they were purified by a reverse-phase HPLC using a ODS column (eluent: 0.1 % AcOH aqueous solution). The pseudo peptides **1a** and **1b** were obtained as acetates and characterized by ^1H NMR, FAB-MS and elemental analysis.[9]

Scheme 1



Scheme 2



(a) DPPA, Asp(OBzl)$_2$•TosOH, Et_3N, DMF; (b) DPPA, Arg(Mts)-OBzl•HCl, Et_3N, DMF; (c) TFMSA, thioanisole, *m*-cresol,TFA; (d) Amberlite® IRA-400 (Cl-); (e) DPPA, D-Arg(Tos)-OBzl•HCl, Et_3N, DMF

Table 1 shows the inhibitory effect of the partially modified retro and retro-inverso pseudo peptides (**1a** and **1b**) on the experimental metastasis of the murine B16-BL6 melanoma.[2, 3] Both of the pseudo peptides showed effective inhibition of tumor metastasis, while the Arg-Gly-Asp-Ser at the same dose did not

show significant efficacy. Although the partially modified retro analog **1a** has opposite chirality against the natural peptide at arginyl residue, it shows comparable anti-metastic efficacy to the partially modified retro-inverso analog **1b** having the same chirality of the natural peptide. Pierschbacher and Ruoslahti revealed that the replacement of L-Arg with D-Arg in Gly-Arg-Gly-Asp-Ser-Pro-Cys was tolerated against the ability of the peptide to inhibit the attachment of cells to a fibronectin substrate.[10] These results suggest that the stereochemistry of the arginyl residue of the RGD peptide is not concerned with recognition of the receptor. To estimate the stability of **1a** against enzymatic degradation in the circulation, time-dependent decomposition of **1a** in plasma was determined at 37°C. The partially modified retro analog **1a** was scarcely degraded during 6h incubation period, but the RGDS peptide was degraded up to 80% in this condition. We speculate that resistance to the enzymatic degradation would prolong life time of the pseudo peptide *in vivo*. As the result, the pseudo peptide would show higher anti-metastatic potency than the RGDS peptide.

Table 1. Effect of Retro and Retro-inverso Peptides of RGD on Experimental Lung Metastasis Produced by i.v. Injection of B16-BL6 Melanoma.

Sample	Dose (μg/mouse)	No. of lung metastasis on day 14 Mean ± SD	(Range)
Untreated (PBS)		101±14	(61-133)
RGDS	500	93±45	(44-154)
retro peptide **1a**	500	19±11	(4- 36)
retro-inverso peptide **1b**	500	38± 3	(19- 63)

Five C57BL/6 mice per group were administered i.v. with the peptide analogs and B16-BL6 melanoma ($7x10^4$) cells. After 2 weeks from administration, tumor colonies in lung were counted.

RGD sequence of the adhesive molecule is also recognized by the glycoprotein IIb/IIIa (GPIIb/IIIa) which appears on the surface of the activated platelet. It is well known that various peptides having RGD sequence inhibit the platelet aggregation. Table 2 shows inhibitory effects of the pseudo peptides and the RGDS peptide on ADP-induced human platelet aggregation *in vitro*.[11] The partially modified retro analog **1a** showed slight inhibitory effect on the platelet aggregation (IC_{50}=2.4 mM), which is one order less potent than the RGDS peptide (IC_{50}=0.18 mM). The partially modified retro-inverso analog **1b** also showed similar low inhibitory activity comparable with **1a**. These results suggest that the partially modified retro and retro-inverso peptides of the RGD peptide show low binding affinity to a GPIIb/IIIa receptor.

Table 2. Inhibition of ADP-Induced Human Platelet Aggregation by Retro and Retro-inverso Peptides

Sample	IC_{50} (mM)	% inhibition at 1mg / ml
RGDS	0.18	100%
retro peptide **1a**	2.4	57%
retro-inverso peptide **1b**	n.d.[a]	53%

a) no data.

In conclusion, the partially modified retro and retro-inverso analogs of the RGD peptide showed higher inhibitory effect on the experimental lung metastasis in mice but lower inhibitory activity on ADP-induced human platelet aggregation comparing with the natural RGDS peptide, which means selective increase on the anti-metastatic activity was obtained by using the pseudo peptides. We are now proceeding to investigate their biological properties in detail.

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9. Based on the elemental analysis, the products will contain some moisture.

   Data for **1a**. ^{1}H-NMR (600MHz, H_2O/D_2O=9/1, pH=6.9, 278K), 1.58-1.68 (m, 2H, Arg γCH_2), 1.72-1.78 (m, 1H, Arg βCH_2), 1.86-1.92 (m, 1H, Arg βCH_2), 1.93 (s, 3H, AcO^-), 2.54 (dd, 1H, J=10.4, 16.0Hz, Asp βCH_2), 2.75 (dd, 1H, J=3.4, 16.0Hz, Asp βCH_2), 3.20 (br s, 2H, Arg δCH_2), 3.30 (d, 1H, J=15.7Hz, CO-CH_2-CO), 3.49 (d, 1H, J=15.7Hz, CO-CH_2-CO), 4.20 (ddd, 1H, J=4.2, 7.4, 8.0Hz, Arg αCH), 4.44 (ddd, 1H, J=3.4, 7.4, 10.4Hz, Asp αCH), 6.59 (br s, 2H, Arg $NH_2CNH_2^+$), 6.90 (br s, 2H, Arg $\omega NH_2CNH_2^+$), 7.50 (br s, 1H, Arg εNH), 8.55 (d, 1H, J=7.4Hz, Asp αNH), 8.58 (d, 1H, J=7.4Hz, Arg αNH); FAB-MS $(M+H)^+$=376; Anal. Calcd for $C_{13}H_{10}O_2N_5 \cdot CH_3COOH \cdot 1/2H_2O$; C%, 40.54; H%, 5.90; N%, 15.76: Found. C%, 39.59; H%, 5.49, N%, 16.08.

   Data for **1b**. ^{1}H-NMR (600MHz, H_2O:D_2O=9:1, pH=6.9, 278K) 1.58-1.70 (m, 2H, Arg γCH_2), 1.72-1.80 (m, 1H, Arg βCH_2), 1.86-1.92 (m, 1H, Arg βCH_2), 1.93 (s, 3H, AcO^-), 2.55 (dd, 1H, J=10.1, 16.0Hz, Asp βCH_2), 2.73 (dd, 1H, J=3.5, 16.0Hz, Asp βCH_2), 3.21 (br s, 2H, Arg δCH_2), 3.34 (d, 1H, J=15.7Hz, CO-CH_2-CO), 3.44 (d, 1H, J=15.7Hz, CO-CH_2-CO), 4.21 (ddd, 1H, J=4.5, 7.5, 8.0Hz, Arg αCH), 4.43 (ddd, 1H, J=3.5, 7.4, 10.1Hz, Asp αCH), 6.55 (br s, 2H, Arg $NH_2CNH_2^+$), 6.93 (br s, 2H, Arg $\omega NH_2CNH_2^+$), 7.43 (br s, 1H, Arg εNH), 8.46 (d, 1H, J=7.4Hz, Asp αNH), 8.47 (d, 1H, J=7.5Hz, Arg αNH); FAB-MS $(M+H)^+$=376; Anal. Calcd for $C_{13}H_{10}O_2N_5 \cdot CH_3COOH \cdot 1/2H_2O$; C%, 40.54; H%, 5.90; N%, 15.76: Found. C%, 40.04; H%, 5.47, N%, 16.12.
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